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GRANT NUMBER DAMD17-95-1-5036

TITLE: Chronic Organophosphorus Exposure and Cognition

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REPORT DATE: August 1998

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command  
Fort Detrick, Maryland 21702-5400

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REPORT DOCUMENTATION PAGE			Form Approved OMB No. 0704-0188	
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1. AGENCY USE ONLY (Leave blank)		2. REPORT DATE August 1998		3. REPORT TYPE AND DATES COVERED Final (15 Apr 95 - 14 Jul 98)
4. TITLE AND SUBTITLE Chronic Organophosphorus Exposure and Cognition			5. FUNDING NUMBERS DAMD17-95-1-5036	
6. AUTHOR(S) Jerry J. Buccafusco, Ph.D.				
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) Medical College of Georgia Augusta, Georgia 30912			8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5400			10. SPONSORING / MONITORING AGENCY REPORT NUMBER	
11. SUPPLEMENTARY NOTES			19990603 071	
12a. DISTRIBUTION / AVAILABILITY STATEMENT Approved for public release; distribution unlimited			12b. DISTRIBUTION CODE	
13. ABSTRACT (Maximum 200 words) Chronic, low-level exposure to acetylcholinesterase (AChE) inhibitor organophosphorus (OP) insecticides or chemical warfare agents produces abnormalities in the function of brain acetylcholine (ACh) neurons, and in humans they may be associated with impaired cognitive function well after withdrawal from such exposure. The purpose of the present study was to identify the severity of cognitive impairment of rats and monkeys following protracted withdrawal from chronic, low-level exposure to the OP agent diisopropylfluorophosphate (DFP). Assessment of spatial learning (water maze task) in rats began 1 - 17 days after completion of either a 14 day once daily DFP (50, 250, or 500 µg/kg) or vehicle treatment regimen. During the 14 day regimen, prior to withdrawal, spontaneous activity and olfactory behaviors were initially suppressed during DFP exposure, effects to which the subjects became tolerant after receiving the "standard" (250 µg/kg dose) regimen. Performance of the spatial memory task was impaired for up to 21 days after withdrawal from the standard DFP regimen. DFP failed to impair the acquisition of the spatial memory task in rats who had previously experienced the task. Performance of a previously well-learned delayed matching task by monkeys was not affected by DFP regimens after withdrawal of the drug. These results in monkeys also complement those showing that the DFP regimen did not alter the performance of rats working a previously well-learned delayed discrimination task. The DFP regimen induced a protracted decrease in the expression of nicotinic and muscarinic (M2) receptors in several brain regions. However, in certain regions of the hippocampus (e.g., CA1 or subiculum) the decreased expression of nicotinic receptors was still evident 3 weeks after withdrawal from DFP. Levels of AChE activity also were slower to recover for this brain region. This decreased rate of enzyme and cholinergic receptor recovery may underlie the protracted impairment of working memory. DFP-induced spatial memory impairment was completely inhibited either by addition of pyridostigmine to the regimen, or by pre-testing (post-regimen) injection of nicotine - suggesting potential lines of therapy.				
14. SUBJECT TERMS Gulf War Syndrome  Diisopropylfluorophosphate			15. NUMBER OF PAGES 95	
Learning Memory Acetylcholinesterase Cerebral Cortex Hippocampus Organophosphate			16. PRICE CODE	
17. SECURITY CLASSIFICATION OF REPORT Unclassified		18. SECURITY CLASSIFICATION OF THIS PAGE Unclassified		19. SECURITY CLASSIFICATION OF ABSTRACT Unclassified
				20. LIMITATION OF ABSTRACT Unlimited

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11/24/88  
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## INTRODUCTION

Acute exposure to toxic levels of organophosphorus (OP) insecticides or chemical warfare agents can result in the overstimulation of both peripheral organs and central regulatory centers which receive innervation from acetylcholine (ACh)-containing neurons. Because of the lipophilic nature of many OP acetylcholinesterase (AChE) inhibitors, there also exists the potential for overstimulation of central cholinergic systems. Less is known regarding the effects of chronic exposure to low-levels of OP AChE inhibitors. However, during such exposure, animals and humans may become tolerant to the acute toxic effects of OP agents, for example, locomotor abnormalities or gastrointestinal disturbances (Brodeur and DuBois, 1975; Russell et al. 1975; Costa et al., 1982). This behavioral tolerance is theorized to reflect the down regulation or adaptation of ACh muscarinic (Schwartz and Kellar, 1983; Schiller, 1979, Aronstam and Buccafusco, 1987; Yang and Buccafusco, 1994) and nicotinic (Schwartz and Kellar, 1983) receptors in cortical, hippocampal, and striatal regions. Although significant tolerance to OP agents has been widely reported, not all behavioral effects of OP administration become tolerant, and those which do, may become tolerant at different rates. Overstreet and colleagues (1974) reported the development of tolerance in the inhibition of operant responding by diisopropyl-fluorophosphate (DFP), but not in unrewarded responding facilitated by DFP. Moreover, different behavioral measures have been shown to tolerate DFP at different rates (Russell et al. 1989) and different AChE inhibitors (DFP vs soman) elicit tolerance in learned avoidance at dramatically different rates (van Dongen and Wolthuis, 1989).

Although receptor mechanisms of adaptation to chronic, low-level enhancement of cholinergic receptor stimulation may protect against some behavioral symptoms of acute OP toxicity, other untoward effects on higher brain functions may persist. In rats treated chronically with either DFP or disulfoton, initial behavioral signs of OP toxicity were reported to subside within the first 3 days of treatment, but whole-brain functional AChE levels and muscarinic receptor density were both reduced by 70% and 28%, respectively, for as long as 14 days after the onset of drug administration (McDonald et al., 1988). Reduced muscarinic receptor density following chronic OP administration (60-70% below controls) normalizes slowly, if at all (Samson et al., 1985). Also, performance of a spontaneous alternation task was shown to be impaired for several days after the behavioral signs of DFP or disulfoton toxicity had subsided (McDonald et al., 1988). Other investigators have reported similar effects of DFP on a delayed matching-to-position, and a spatial navigation learning task (Abdulla et al., 1993; Bushnell et al., 1991). In both studies, learning was impaired for several days after overt signs of drug toxicity had subsided. Notably, several AChE inhibitors have been demonstrated to produce alterations in the human and primate electroencephalogram which last more than one year (Metcalf and Holmes, 1969; Burchfiel et al., 1976; Duffy et al., 1979; Hoogendam et al., 1962). These data, as a whole, indicate that chronic OP exposure produces significant impairment of learning and memory, and reductions in cholinergic receptor density well after behavioral tolerance has developed.

Given the possibility of neurotoxicity with chronic OP exposure (Samson et al., 1985), and of acute impairment of learning processes, it is possible that a protracted phase of impairment of

certain cognitive processes may result from such exposure. Indeed, workers chronically exposed to OP agents present with a variety of psychiatric sequelae, including, depression, apathy, irritability, and even schizophreniform illness has been diagnosed. One predominate set of symptoms includes loss of concentration, difficulty in thinking, and, especially, memory impairment (Gershon and Shaw, 1961; Metcalf and Holmes, 1969). It is intriguing that many of these symptoms parallel those reported as troubling Persian Gulf Illness patients. Although the neuropathological basis for this protracted cognitive impairment is unknown, it is not likely that the insult represents a severe pathological event as may be observed in idiopathic neurogenerative disorders such as Alzheimer's disease. Rather, it is more likely that accidental exposure to OP agents results in more subtle neuropathological and behavioral effects.

Whereas subtle impairing effects on the highest elements of human cognition may not be difficult to ascertain, in experimental animals this presents a daunting task. There are limited examples in the literature regarding the subtle or selective effects of OP exposure on animal behavior. Thus, impaired memory induced by chronic OP administration appears to be most evident on novel learning tasks (i.e. those which require the greatest reliance on working memory). For example, Gardner and co-workers (1984) reported that, following several injections of DFP, initial passive avoidance training was impaired. Spatial learning in mice was shown to be impaired only if conducted after completion of a chronic DFP administration regimen (Upchurch and Wehner 1987). Performance of this task was not disrupted by chronic DFP administration if animals had been trained prior to the start of DFP administration. Moreover, Bushnell and colleagues (1991) reported that delayed matching-to-position accuracy by rats was impaired during chronic administration of DFP, but that soon after withdrawal of the drug, matching performance returned to baseline (pre-drug) levels. Therefore, memory-related tasks that are well learned prior to OP exposure may be impaired only during drug administration, whereas those tasks that require new learning after the OP regimen may be impaired after drug withdrawal. Hymowitz and co-workers (1990) reported that chronic sub-toxic administration of soman to rats resulted in a gradual reduction in performance of an operant task. Although the rats were generally asymptomatic with respect to AChE inhibition-related toxicity, significant neuronal degeneration was observed. The level of neuropathology under these conditions was more subtle than that resulting from near lethal doses of soman; but was consistent with the subtle nature of the loss of a learned behavior. The results of the above studies (and those featured in the following report) support the possibility that chronic exposure to OP agents can result in long-term cognitive deficits associated with neurochemical alterations, even when overt symptoms of excessive cholinergic activity are not present.

The purpose of the present study was to (1) examine the development of behavioral tolerance to chronic, low-level DFP exposure; (2) determine the extent of memory-related task impairment at several time intervals after DFP withdrawal; (3) characterize the type of memory/cognitive impairment; (4) correlate specific alterations in cholinergic neurochemical markers in specific brain areas with the onset of behavioral changes; and (5) determine whether post-DFP administration of nicotine, a memory enhancing agent, could restore task performance; (6) examine the potential protective effects of pyridostigmine on cognitive outcome after DFP, and

whether a new natural product *celastrus peniculatus* could reverse deficits in muscarinic receptor function.

## METHODS

### *Subjects (Rat studies)*

Male Wistar rats (Harlan Sprague-Dawley), approximately four months old (weighing 350-400 g) were used. Each rat was housed individually in a stainless steel mesh cage in a temperature controlled room (25°C) with free access to food (NIH-07 formula) and water, and maintained on a 12-hour light/dark cycle (lights on at 1800 h). All animal protocols were previously approved by the institutional Committee on Animal Use for Research and Education.

### *Drug administration*

DFP (Sigma, St. Louis, MO) dissolved in saline, ecothiophate iodide dissolved in saline, or saline alone was administered (s.c.) daily for 14 consecutive days in a dose of 50µg/kg, 250µg/kg, or 500µg/kg for DFP and 2.50µg/kg for ecothiophate (N= 10-12 for each dose). All injections were given in a volume of 1ml/kg body weight between 0900 and 1100 h.

### *Observational Analysis*

Immediately after each rat's daily DFP injection (see below) he was placed in a clear polypropylene chamber (25 x 45 x 25 cm) for 35 min. After an initial 5 min acclimation period, olfactory reactivity (rearing and sniffing) was recorded for 10 min. In addition, animals were weighed and the occurrence and/or frequency of other indicators of OP toxicity were recorded, including: tremor, salivation, diarrhea, lacrimation, and urination. At the end of the 30 min observation period, animals were returned to their home cages and no further observations were made until the following day. After the first experimental series, observation of specific locomotor behaviors over the course of the DFP regimen was not systematic since the behavioral pattern was characteristic and reproducible.

### *Water Maze Testing*

Maze testing was performed in a circular pool (diameter: 180 cm, height: 76 cm) made of plastic (Bonar Plastics, Noonan, GA) with the inner surface painted black. The pool was filled to a depth of 35 cm of water (non-opaque, maintained at 25°C) which covered a black 10 cm square platform. The platform was submerged 1 cm below the surface of the water and placed in the center of the northeast quadrant on all trials. The pool was located in a large room with a number of extra-maze visual cues including highly reflective geometric images (squares, triangles, circles, etc.) mounted on the wall. Diffuse lighting and black curtains were used to hide the experimenter and the awaiting rats. Swimming activity of each rat was monitored via a ccTV camera mounted overhead, which relayed information including latency to find the platform,

time and distance spent in each quadrant, and swim speed (latency/distance traveled), to a video tracking system (Poly-Track, San Diego Instruments, San Diego, CA).

**Hidden Platform Test:** Each rat was given four trials per day (session) for 4 consecutive days. On days 1- 4, a trial began by placing the rat in the water facing the pool wall in one of the four quadrants (designated northeast, northwest, southeast, and southwest). Rats were not allowed to acclimate to the pool prior to testing at any point during days 1- 4. The daily order of entry into individual quadrants was randomized such that all 4 quadrants were used once every day. For each trial, the rat was allowed to swim a maximum of 90 sec in order to find the hidden platform. When successful, the rat was allowed a 30 sec rest period on the platform. If unsuccessful within the allotted time period, the rat was assigned a score of 90 sec, and he was physically placed on the platform and allowed the 30 sec rest period. In either case, the rat was given the next trial (intertrial interval = 30 sec) after the rest period.

**Acquisition Phase:** Different groups of animals (N=10-12 per group) were used to assess the affects of low-level DFP exposure on ability to initially learn the hidden platform task and to assess the effects of such exposure on ability to recall previously learned task navigation strategies. In the Acquisition Phase, two groups of animals were used. Groups received daily s.c. injections of saline or DFP in saline (0.25 mg/kg) for 14 consecutive days. During this period, water maze testing was not conducted. This dose of DFP was chosen based upon the results of our first experimental series, and for the purposes of this report is termed the standard DFP regimen. Seven days following the last injection of DFP, all animals were tested in the water maze hidden platform test for 4 consecutive days, as described above.

**Relearning Phase:** During a relearning phase of water maze testing, an animal's ability to locate the hidden platform may be re-assessed after delays of variable length. In the present study, after different groups of animals completed the initial 4 days of maze training they were administered DFP for 14 consecutive days (standard regimen) without maze exposure, allowed to withdraw from DFP exposure for 7 days and then tested in the water maze for 4 consecutive days in a manner identical to that used during training.

#### *Delayed Stimulus Discrimination Task (DSDT)*

Male Wistar rats (N=12 per group), 45 days old, were obtained from Harlan Sprague-Dawley, Inc. and housed individually in stainless steel mesh cages in a temperature controlled room (25°C) with a 12-hour light/dark cycle. Upon arrival, each animal was provided with water and standard rodent chow (NIH-07 formula) *ad libitum* for one week. They were restricted during the next week to a daily feeding of 18 grams per day (approximately 80% of their *ad libitum* consumption). Additional food was given on weekends and holidays to maintain the weight of each rat at approximately its freely fed weight. Rats were trained and tested in operant chambers that were enclosed in ventilated, sound and light attenuated cubicles (dimensions, 27.9 x 29.2 x 30.5 cm, Coulbourn Instruments, Allentown, PA). The operant chambers were manufactured, and the operational software was developed "in house" by the staff of the Department of



Biomedical Engineering, of the Medical College of Georgia. Dim chamber illumination was provided by a small 3 watt house light located on the cubicle ceiling. Each chamber was fully computer automated with levers centered on each side of a feed box. Each lever, one on the right and one on the left, could be depressed to earn food pellet rewards. The rats were trained 5 days per week, Monday through Friday. Beginning two weeks after arrival, they were trained to lever press (without a presented stimulus) on a 30-trial continuous reinforcement schedule (45 mg pellets, Bioserv) in which the doors were always open and either lever press was rewarded. Once a particular rat was routinely successful at obtaining 30 pellets, it was graduated to a program in which 32 each of the light and tone configurations were randomly distributed throughout 64 trials.

The animals were then trained to discriminate between the light and the tone, i.e., the reward was provided only on the right side following a trial which began with the presentation of a light, whereas following trials beginning with the presentation of a tone, only a response to the left side was rewarded. The duration of each stimulus was 3 seconds. During the stimulus presentation and during an imposed delay, rats were sequestered behind retractable doors which remained closed to prevent access to the levers. At the end of the delay, the doors quickly opened, allowing access for lever selection by the rat. The doors remained open for 5 sec to allow time for the rat to choose a lever and if a correct choice was made, consume its reward. Finally the doors were gently closed for a total inter-trial interval of 10 sec. If an incorrect choice was made, no reward was given and the next trial was initiated. The initial delay period was 1 sec. When accuracy for a given animal averaged between 85 and 95% correct for a period of approximately 2 weeks, the delay was increased to 3 and then to 5 sec when accuracy again approached 90%. Rats were maintained on 5 sec delays for 14 weeks and daily performance averaged between 80% and 85% correct prior to the onset of DFP administration.

#### *Brain AChE Assay*

Rats were sacrificed by decapitation and brains were removed, frozen in liquid nitrogen, and stored at  $-70^{\circ}\text{C}$  until assayed. Brains were taken at 3 separate time points following the end of DFP administration: (1) 3 days after the last injection (corresponding to the start of behavioral testing for other animals); (2) immediately after completion of the last day in the first phase of water maze training (7 days after the last injection); or (3) immediately after completion of the last day in the second phase of water maze training (21 days after the last injection). Brain regions (frontal cortex and hippocampus) were dissected out and assayed spectrophotometrically in phosphate buffer at pH 7.9 after Ellman et al. (1961). Homogenized brain regions (25%) were introduced into a cuvette containing the reaction mixture (7.5 nM acetylthiocholine iodide substrate and 6.9 mM dithiosbenzoic acid. Absorbance at 412 nm was recorded for 4 min. Protein concentrations were spectrophotometrically measured using the Bio-Rad Protein Assay (Richmond, CA) system and using bovine albumin as standard.

#### *Receptor Autoradiography*

Separate groups of rats were examined at 3, 7, and 21 days after OP discontinuation. Immediately after decapitation the brain was removed and flash frozen in dry ice/isopentane. The frozen tissues were stored at -70°C until use. The procedures we use for quantitative receptor autoradiography have been described in detail elsewhere (Wei et al., 1995). Briefly, each frozen brain was sectioned coronally from the frontal pole through the level of the cerebellar peduncles. Autoradiographic conditions are summarized below:

<u>Receptor</u>	<u>Ligand</u>	<u>Isotope</u>	<u>Buffer</u>	<u>Preinc</u>	<u>Incubation</u>	<u>Exposure</u>
nicotinic	epibatidine	<sup>3</sup> H	Tris-Hepes	30 min; 4°C	120 min; 4°C	4-6 weeks
muscarinic	QNB	<sup>3</sup> H	Tris-phosphate	30 min; 25°C	120 min; 25°C	2 weeks
muscarinic M2	AFDX 384	<sup>3</sup> H	Tris-phosphate	30 min; 25°C	120 min; 25°C	2 weeks

Receptor binding in specific brain nuclei was quantified using NIH Image software. Receptor quantification was performed for each brain region that expressed a signal greater than background. Molar quantities of ligand bound were determined using values interpolated from the optical density versus a tissue radioactivity standard curve. Each structure was measured bilaterally in at least four sections for each animal. Binding analyses for each ligand were performed in consecutive sections from the same brain sample. Because autoradiographic films were not all developed together (because of the sample size) the binding data were normalized with regard to respective controls in each assay.

#### *Acetylcholine vesicular transporter mRNA levels*

Rats were injected (sc) with (-)-nicotine (0.8 mg/kg) as the bitartrate salt (Sigma) 1 hr before decapitation. The dose of (-)-nicotine was chosen based on a previous report that a single injection of this dose produced increases in expression of mRNA encoding tyrosine hydroxylase *in vivo* (Mitchell et al. 1993). Rats were sacrificed by decapitation and their brains were removed and rapidly dissected at 4°C. Cerebral cortex and hippocampus samples were then flash frozen in liquid nitrogen and stored at -70°C until assay. Nondegraded total RNA was isolated from each sample using the chloroform-phenol based FastRNA-Green kit developed by Bio 101, Inc. (Vista, CA). Pelleted RNA fractions were suspended in water and stored at -70°C.

RT-PCR: Oligonucleotides designed to amplify a 440 bp segment of the VACHT mRNA sequence (Genebank # 507745) were purchased from the Program for Critical Technologies in Molecular Medicine (Yale University, New Haven, CT). Oligonucleotide sequences and corresponding base sites were as follows: 5'-CTG GTG CTG GTC ATC GTG TG-3' (upper primer, base position 958) and 5'-GCG AAG AGC GTG GCA TAG TC 3' (lower primer, base position 1,378). The sequences of oligonucleotides were developed using the Oligo Program (National Bioscience, Plymouth, MN). Murine Leukemia virus Reverse Transcriptase (2.5 U;

Perkin Elmer, Norwalk, CT) and priming with random hexamers (2.5 $\mu$ M) were used to synthesize cDNA from RNA in a reaction volume of 20 $\mu$ l. All RNA samples were reverse transcribed and cDNA amplified in duplicate. The PCR reaction volume (100  $\mu$ l) also contained 5mM MgCl<sub>2</sub>, 50mM KCl, 10 mM Tris HCl, and 1 mM deoxynucleotide triphosphate precursors at pH 8.3. The reverse transcription step was omitted in 1 PCR tube from each RNA sample to check for the presence of contaminating genomic DNA. cDNA was amplified for 30 cycles of 95°C for 1 min and 62°C for 1 min using the GeneAmp RNA PCR kit (Perkin Elmer, Norwalk, CT) and a Perkin Elmer Cetus model 480 thermal cycler. After amplification, aliquots of the cDNA (20  $\mu$ l) were loaded onto ethidium bromide-stained agarose gels (1.5% agarose) and electrophoresed at 75 kV for 1 hr to confirm the molecular weights of products. One lane of each gel contained a 100 bp ladder molecular weight standard sample (Gibco BRL, Gaithersburg, MD). PCR products were visualized on gels with UV illumination (254nm) and photographed. A restriction enzyme digest (*PST* I) has previously been performed on the VACHT RT-PCR product described and produced the predicted restriction fragments, based on the derived sequence of the 440 bp cDNA fragment (Prendergast et al. 1996).

High Pressure Liquid Chromatography: Aliquots (15 $\mu$ l) of RT-PCR products were used for HPLC analysis without further purification. The HPLC system consisted of Bio-Rad model 1350 pumps and a model 1706 UV-visible monitor (Richmond, CA). The analytical column (TSK DEAE-NPR; Perkin Elmer) was packed with 2.5 nm diameter particles of hydrophilic resin bonded with diethylaminoethyl groups. The mobile phase consisted of reservoir A containing 1 M NaCl and 25 mM Tris HCl, pH 9.0 and reservoir B containing 25 mM Tris HCl, pH 9.0. The gradient used was 46-54% A for 0.1 min, 54-60% A for 3.9 min, 60-75% A for 1 min, and 75-100% A for 5 min followed by 100-46% A for 3 min. The column was operated at a flow rate of 1 ml/min at room temperature and the UV monitor was detecting at 254 nm. The relative amounts of RT-PCR products were determined as the area under the peak in arbitrary UV absorption units (a.u.) of VACHT product. Each data point of RT-PCR product represents the mean of duplicate RT-PCR assays for a given rat and a given brain region. Differences in the relative abundance of RT-PCR product in nicotine treated and control animals was compared for each brain region using analyses of variance.

#### *Subjects (Non-human primate studies)*

Four young macaques (1 male *macaca nemestrina*; 2 male *macaca mulatta*; and 1 female *macaca mulatta*) served as subjects. Monkeys were individually housed at the Animal Behavior Center of the Medical College of Georgia in stainless steel cages composed of multiple 50 x 28 x 26 in units. Toys and foraging tubes were provided routinely and monkeys were allowed to observe television programs each afternoon after testing to promote psychological well-being. During a test-week, monkeys were maintained on a feeding schedule which allowed approximately 15% of their normal daily food intake to be derived from banana-flavored reinforcement pellets awarded for correct responses during testing. Standard laboratory monkey chow, fresh fruits, and vegetables comprised the remainder of their daily food intake. Water was available *ad libitum*. All procedures used during this study were reviewed and approved by the

Medical College of Georgia Institutional Animal Care and Use Committee and are consistent with AAALAC guidelines.

#### *DFP administration in primates*

DFP was dissolved in sterile phosphate (5 mM) buffered saline (pH 6.0) and administered daily in the gastrocnemius muscle within a volume of 0.035 ml/kg body weight. A test session began 10 min following drug or saline administration. In one preliminary experiment, a monkey was administered a dose of 50 µg/kg for 7 days, at which time toxicity necessitated withdrawal of DFP. Three additional monkeys initially received daily administration of 10 µg/kg DFP for a period of 31 consecutive days. Because of the lack of a treatment response, the daily dose was increased to 15 µg/kg for the subsequent 15 days. Given a continued lack of effect on DMTS accuracy, the daily dose was again increased, to 20 µg/kg. During 10 days of administration of this dose of DFP, significant symptoms of chronic OP toxicity (e.g. diarrhea, muscle fasciculations, and muscle weakness) became increasingly evident in all monkeys. After 10 days (56 days after the start of DFP administration) administration of the regimen of DFP was discontinued, but daily DMTS testing was continued.

#### *DMTS Procedure*

For DMTS testing, test panels were attached to the home cages. Stimuli on the test panels were 2.54 cm diameter colored disks (red, yellow, or green) presented by light-emitting diodes located behind clear plastic push-keys. A trial began with the illumination of the sample key by one of the colored disks. A key-press extinguished the sample light and initiated one of four pre-programmed delay intervals, during which no disks were illuminated. Following the delay interval, two choice lights located below the sample key were illuminated. One of the choice lights matched the hue of the sample light. These disks remained illuminated until a monkey pressed one of the two lit keys. Key-presses of choice stimuli which matched the hue of the sample stimulus were rewarded by a 300 mg banana-flavored pellet. Non-matching choices were neither rewarded nor punished. Matching configurations were fully counterbalanced for side, delay, and hue. A new trial was initiated 5 sec after the second key-press on a preceding trial. Monkeys completed 96 trials on each day of testing.

Four possible delay intervals between a monkey's response to the sample light and the presentation of the two choice lights were employed: zero sec delay and a short, medium, and long delay. Short, medium, and long delay intervals were individually adjusted to produce stable performance levels approximating the following performance levels: short (75-84% correct); medium (65-74% correct); and long (55-64% correct). Monkeys performance for zero sec delay trials averaged 85-100% correct. The rationale for this procedure was to normalize performance based on the widely varying capabilities of the monkeys (Buccafusco et al., 1995). The following parameters were recorded during all test sessions: percent correct at each of four delay intervals and latency of response to sample and choice stimuli.

### *Erythrocyte AChE Assay*

Heparinized control blood samples were obtained from a femoral artery or vein of each monkey prior to the start of initial DFP administration. Additional samples were obtained at multiple time points after the start of DFP administration depending on the presence of toxic symptoms in the monkey. Plasma was separated from red blood cells by centrifugation (2500 rpm, 10 min) and frozen (-70°C) until assayed. Erythrocyte AChE activity was assessed as described above.

### *Statistics*

During maze acquisition, data were collapsed across trials for each day and averaged to obtain a mean latency for each animal. A two-way analysis of variance with *post hoc* Newman-Kewls comparisons were used to compare daily group latencies during days 1-4 of training, as well, as data from the transfer test, visual acuity, and swim speeds. Two-way ANOVA was used to compare AChE activity in the different brain regions examined for each time point of tissue collection. Frequency of locomotor behaviors and olfactory behavior were analyzed using the nonparametric Kruskal-Wallis test. For data derived from the primate studies, a two-way ANOVA that included delay interval and day of testing as within subjects factors was used to examine the effects of DFP administration on DMTS accuracy throughout 56 days of drug administration. To minimize variability, data were groups as epochs of 5 consecutive days of behavioral testing and performance on each delay interval for a given 5 day period was averaged to yield a single percentage correct for a given delay during a given epoch.

## **RESULTS**

### **Characterization of the DFP Regimen in Rats**

#### *Spontaneous Locomotion, Olfactory Reactivity, and Body Weight*

The frequency of both spontaneous locomotor activity and olfactory behavior (rearing and sniffing) were recorded during the 30 min period immediately following DFP or saline administration on each day of administration (with the exception of weekend days). In rats which received saline or the lowest dose of DFP (50 µg/kg), olfactory behavior was stable across 10 days of observation. Beginning on the third day of drug administration, however, the rats that received 250 µg/kg or 500 µg/kg of DFP displayed significantly less olfactory reactivity than did controls or animals treated with the lowest dose of DFP ( $H = 14.6$ ,  $p < 0.01$ ). With the 250 µg/kg dose, olfactory behavior was suppressed until Day 6, at which time, behavioral tolerance to this dose was evident. From Day 7-Day 10, these animals displayed olfactory behavior at levels similar to controls (Fig. 1). Animals that received the higher dose of DFP (500 µg/kg), however, remained suppressed in their olfactory behavior throughout testing. Each group of rats, with the exception of those that received the 50 µg/kg dose of DFP, exhibited a reduction in spontaneous locomotion from Day 1 to Day 2, possibly related to the novelty of the testing

environment (Fig. 2). Those that received the two higher doses were significantly less active on Days 2 - 4 than were controls or rats that received the lowest dose ( $H = 23.6$ ,  $p < 0.001$ ). As with olfactory behavior, those which received the 250  $\mu\text{g/kg}$  dose returned to control levels of behavior by Day 6 of testing. With the 500  $\mu\text{g/kg}$  dose, locomotor behavior continued to decline and was absent in nearly all animals by Day 6. In contrast, those rats that were treated with the 50  $\mu\text{g/kg}$  dose of DFP displayed significantly more locomotor activity than did controls on Days 7-10 ( $H = 33.70$ ,  $p < 0.001$ ).

In addition to DFP-induced behavioral alterations (reflected in locomotor and olfactory behavior) the rats treated with the highest dose appeared to exhibit anorexia which resulted in significant losses in body weight (Fig. 3). A two-way ANOVA examining drug treatment and day of administration revealed a significant interaction between dose of DFP and day of administration [ $F(3,152) = 39.69$ ,  $p < 0.001$ ]. *Post hoc* analysis indicated that, with the highest dose of DFP, body weights decreased significantly from Days 4-10 of drug administration, as compared to the change in body weight of controls ( $p < 0.05$ ). By Day 10 of drug administration, the weight of these rats averaged  $73.00 \pm 11.58$  g lower than that prior to the first drug administration. In contrast, body weight was significantly increased by administration of the 50  $\mu\text{g/kg}$  dose of DFP. Increases in body weight were evident from the onset of drug administration and were significantly elevated as compared to all groups on Days 7-10 ( $p < 0.05$ ).

Approximately 40% of those animals that received the highest dose of DFP (500  $\mu\text{g/kg}$ ) displayed muscular fasciculations, weakness, and anorexia sufficient to cause death or warrant euthanasia. However, signs of severe OP toxicity (e.g. fasciculations, lacrimation, dyspnea, diarrhea) were not observed in most rats. For example, in this initial study, of those rats that received the 250  $\mu\text{g/kg}$  regimen, about 10% displayed a similar and severe response to DFP. In both groups, the severely affected rats died or had to be euthanized between 10 and 14 days after the start of DFP administration. Over the course of the entire study, we observed that between 0-2 animals per study group (20-24) would not survive the DFP regimen.

### *Spatial Navigation Learning*

Rats in each treatment group demonstrated the ability to eventually learn the water maze task during the 4 days of testing (Fig. 4). A main effect for day (session) of testing was observed independently of drug treatment [ $F(3,87) = 41.82$ ,  $p < 0.001$ ]. *Post hoc* analysis indicated that, in all groups, performance of the water task improved significantly on each consecutive session of testing ( $p < 0.05$ ). However, the rate of learning was markedly different for the groups. During the first session of testing, starting 2 days after the final DFP or saline injection, animals which received the 250  $\mu\text{g/kg}$  dose of DFP were impaired in their ability to find the hidden platform. A significant effect for drug treatment (saline vs dose of DFP) was observed, indicating that these animals displayed latencies to find the platform which were significantly greater than were those of controls or animals which received the 50  $\mu\text{g/kg}$  dose of DFP across all days of testing [ $F(2,87) = 4.53$ ,  $p < 0.05$  for Newman-Keuls *post hoc* comparisons]. Rats that received the 50  $\mu\text{g/kg}$  dose displayed latencies similar to those of controls. Administration of the 250  $\mu\text{g/kg}$

dose resulted in increased latencies of 18.0sec, 13.7sec, and 13.0 sec greater than controls on Days 1,2, and 3 of training and testing, respectively. This occurred independently of overt drug toxicity (e.g. muscle fasciculation, lacrimation, diarrhea, etc) in that swim speeds and visual acuity were unaffected by any dose of DFP (Table 1).

To examine the possibility that impaired learning following DFP exposure was associated with peripheral AChE inhibition, the peripherally acting quaternary AChE inhibitor echothiophate iodide was administered daily in a dose of 2.50 $\mu$ g/kg for 14 days and animals were tested beginning 2 days after the last injection. The dose was chosen based on our observation that this dose suppressed plasma butyrylcholinesterase in a manner similar to that of the 250  $\mu$ g/kg dose of DFP (to only 45-50% of control levels). This treatment regimen with echothiophate had no significant effect on spatial navigation learning (Fig. 4), swim speed, or visual acuity.

Additional groups of rats (N= 10 per group) administered saline or the 250  $\mu$ g/kg standard DFP regimen for 14 days were not tested in the water maze until 3 (Fig. 5) or 17 (Fig. 6) days after the last dose was injected. Even at the 17 day measurement, water maze performance continued to be impaired by DFP (although this effect was independent of session) [ $F(1,36) = 4.67$ ,  $p < 0.05$ ]. For animals that received DFP and that were tested after 17 days, latencies to find the platform during sessions 1-4 were 16.6 sec, 8.3 sec, 14.3 sec, and 26.8 sec greater than controls, respectively (Fig. 6). In fact, the latencies of animals treated with DFP were significantly greater than those of saline-treated rats during all 4 sessions of testing, particularly on Day 4. This effect of DFP may be compared to those of the previous experimental groups (that began testing only 2 or 3 days after the last dose of DFP) for which water maze learning had achieved control levels of performance as soon as Day 3 or 4 (Fig. 4 and 5). All rats, regardless of treatment, began to acquire (learn) the task with repeated exposure over at least the first 3 sessions of testing (main session effect = [ $F(3,36) = 10.19$ ,  $p < 0.001$ ]). That is, although DFP treated rats were impaired relative to controls in task performance, overall there was a significant session dependent decline in latencies (improvement in task performance). *Post hoc* analysis indicated that for all rats, the latency to find the platform on the training day was significantly greater than on sessions 2 and 3 ( $p < 0.05$ ). At this time point, no effect of DFP on swim speed, dwell time, or visual acuity was observed, indicating the absence of drug-induced side effects.

#### *Brain AChE Levels in DFP Treated Rats*

Groups of animals (N=10) were sacrificed 3, 7, or 21 days after completion of the 14 day DFP treatment (250  $\mu$ g/kg) regimen and AChE activity in both the frontal cortex and hippocampus was examined (Table 2). These time points were chosen to correspond with the start of initial testing in the water maze and with the completion of water maze testing (Day 5) at each time point. Three days after the last DFP administration, AChE levels were significantly suppressed in both the cortex and hippocampus ([ $F(1,15) = 207.55$ ,  $p < 0.001$ ]). Enzyme activity was suppressed by DFP to 42.6% and 50.4% of controls in each of these regions, respectively. Moreover, baseline AChE activity was different between the two regions with the hippocampus exhibiting a 15% higher level of enzyme activity above cortex levels [ $F(1,15) = 17.84$ ,  $p <$

0.001]. At the 7 day point cortical enzyme levels had recovered to 81.9% of control levels. However, hippocampal AChE activity had recovered to only 64.6% of control levels (Table 2). Twenty-one days after the final DFP injection, AChE activity in both regions had returned to control levels and no significant differences in enzyme activity attributable to DFP treatment were observed (Table 2).

### **Characterization of the DFP regimen in monkeys**

#### *Effect of DFP on the performance of the DMTS task by monkeys*

For one monkey (a mature male *macaca nemestrina*), the selected starting dose was 0.05 mg/kg of DFP. This dose was chosen based on previous data indicating that a ten-fold higher dose (50 µg/kg) was the highest dose tolerated chronically by rats (Poirier et al., 1977). It was estimated that significant AChE inhibition would occur but that overt symptoms of toxicity would not. He was administered this dose once daily for 7 consecutive days and failed to complete the DMTS task beginning on the second day of DFP administration. Overt signs of DFP toxicity (diarrhea and lethargy) were evident beginning 3 days after the start of DFP administration and warranted discontinuation of drug after 7 days. (During this study 0.4 mg, i.m. atropine methyl bromide was administered once daily to each monkey that exhibited diarrhea until the symptoms were controlled.) This monkey failed to complete DMTS testing during the subsequent week and his participation in the study was terminated. For the subsequent 14 days, this monkey completed no more than 11 of the 96 DMTS trials on any day of testing. By 15 days after withdrawal, he began completing all 96 trials and DMTS accuracy was at levels similar to those observed prior to DFP administration. In addition, all overt symptoms of toxicity had subsided by this time. No persistent effects of drug, including those which may be indicative of NTE inhibition, were observed.

Pretreatment of the three rhesus monkeys with saline resulted in the following level of baseline DMTS accuracy for each delay interval: 0 sec delay =  $94.9 \pm 0.94\%$ ; short delay =  $82.5 \pm 2.1\%$ ; medium delay =  $70.7 \pm 1.97\%$ ; and long delay =  $66.0 \pm 2.5\%$ . These monkeys began DFP administration at a dose of 10 µg/kg. During 25 days of testing conducted daily after administration of this dose, DMTS accuracy for each delay interval was not significantly different from baseline pre-drug accuracy (Fig. 7). Similarly, during 15 consecutive days of administration of the 15 µg/kg dose, DMTS accuracy was not significantly affected. A non-significant trend towards a decrease in accuracy on medium and long delay trials was observed, however, during periods 8 and 9 (days 10-15 of administration for the 15 µg/kg dose) of DFP administration. During the initial 5 days of exposure to the 20 µg/kg dose of DFP, accuracy for all delay intervals remained at or slightly below baseline levels. However, within this 5 day period symptoms of DFP toxicity (e.g., daily mild diarrhea- all 3 monkeys, muscle fasciculations- 1 monkey) became increasingly evident. No other symptoms of OP toxicity were observed. The severity of symptoms warranted the discontinuation DFP administration. During the five day testing period immediately following the end of DFP administration, DMTS accuracy appeared to return to levels similar to those obtained during baseline testing. Sporadic



symptoms of OP toxicity (diarrhea and muscle fasciculations) persisted, however, for approximately 15 days after withdrawal from DFP administration.

Latencies to respond to sample stimuli were unaffected by DFP administration (Table 3). Choice response latencies differed on correct and incorrect trials [ $F(1,16) = 57.53$ ,  $p < 0.05$ ]. Latencies to respond to sample stimuli on correct and incorrect trials were not affected by administration of any dose of DFP (Table 3). At no time during testing did monkeys display tendencies to adopt non-mnemonic strategies in that no side (left vs. right choice key) and/or stimulus color (red, yellow, or green) preferences were observed during baseline testing or during DFP administration. For side preferences, left and right keys were chosen on an equal basis of 50-57% throughout testing. The three color choices were also chosen equally, averaging 30-36% chosen for each color throughout testing.

#### *Erythrocyte AChE Levels in DFP Treated Monkeys*

In the pigtail monkey who received the 50  $\mu\text{g/kg}$  dose of DFP for 7 consecutive days, AChE enzyme activity was suppressed to only 4.8% of baseline by 14 days after the start of DFP treatment (7 days after withdrawal from DFP). Recovery of enzyme activity was monitored in this monkey for 76 days following withdrawal of DFP treatment, at which time, AChE activity had recovered to only 63.3% of baseline. For each of the 3 rhesus monkeys, erythrocyte AChE levels were obtained prior to the start of DFP administration and 14 days after the start of administration of the 10  $\mu\text{g/kg}$  dose of DFP to confirm AChE inhibition (Table 4). Baseline AChE for the 3 monkeys averaged  $0.0133 \pm 0.0007$   $\mu\text{mol}$  of substrate hydrolyzed/min/mg protein. Following 14 days of treatment with the 10  $\mu\text{g/kg}$  dose of DFP, AChE activity was suppressed to only 0.0032  $\mu\text{mol}$  of substrate hydrolyzed/min/mg protein, a significant decrease in activity from baseline [ $F(1,2) = 247.80$ ,  $p < 0.01$ ]. AChE activity at this time point represents a decrease to only  $23.74 \pm 3.33\%$  of baseline enzyme activity. Additional blood samples were not collected during DFP administration so as to avoid disruption of ongoing DMTS performance and to avoid possible toxic interactions (eg. respiratory difficulty) between chronic DFP treatment and anaesthetics. Furthermore, given the lack of effect of DFP on DMTS performance and the previously reported lack of correlation of enzyme activity with learning and memory, additional assays of AChE activity were not performed.

#### **Effect of DFP on Rats Performing the Delayed Stimulus Discrimination Task (DSDT)**

Prior to the onset of DFP administration, accuracy on 5 sec delay trials averaged between 83.0 and 83.6 % correct for all animals tested after saline administration on three occasions separated by 7 days (Fig. 8, inset). During the initial 2 days of Relearning in this model, DSDT accuracy remained near this level, averaging 77.5 and 74.0 % correct for saline- and DFP-treated animals, respectively (Fig. 8). No significant differences between these groups were observed on any of the five days of testing ( $p < 0.33$ ). However, all animals demonstrated an increase in DSDT accuracy during the 5 day testing period [ $F(4,92) = 4.87$ ,  $p < 0.01$ ]. Performance was elevated, relative to Days 1 and 2, for each of Days 3, 4, and 5. *Post hoc* analysis indicated that on Days 3

and 5, DSDT accuracy was significantly greater than on Days 1 and 2 for all animals, independent of drug treatment group ( $p < 0.05$ ).

### **Effect of DFP on the performance of rats relearning the Spatial Navigation Learning task**

#### *Acquisition Phase*

The rats in each treatment group exhibited progressive improvement in task performance over the four consecutive days (sessions) of testing (Fig. 9). However, the rats that received the standard DFP regimen (250  $\mu\text{g/kg}$ ) were impaired in their ability to locate the hidden platform during sessions initiated on Days 2-4 of testing, relative to control animals ( $F(3,54) = 4.64$ ;  $p < 0.01$ ). The results of this experiment serve simply to confirm the effect of the DFP regimen that we obtained in earlier experiments (Figs. 4 - 6).

#### *Relearning Phase*

In a separate study group, each rat received a complete 4 day water maze experience. These subjects performed the task in a manner similar to that of our earlier saline control groups as they demonstrated the ability to eventually re-locate the hidden platform during 4 days of testing (Fig. 10, inset). With each consecutive day of training, latencies to find the platform were significantly reduced, as compared to the first day of training [ $F(3,18) = 9.39$ ,  $p < 0.001$ , *post hoc* analysis =  $p < 0.05$ ]. The study group was divided into those that received saline and those that received the standard 14 day DFP regimen. Seven days after the withdrawal from DFP (or saline) regimen, all animals were subjected to a second 4 day program of water maze training (the Relearning phase). On the initial day of Relearning, control animals demonstrated latencies to find the platform of 30-35 sec, a reduction from mean of 48 sec during Day 1 of initial water maze testing (Fig. 10). Thus, there appeared to have been retention of spatial navigation strategies. However, the animals as a whole did not perform as well as they did during the final trials of the initial training period. All rats, independent of treatment condition, demonstrated a progressive learning experience during the course of 4 days of Relearning [ $F(3,54) = 23.35$ ,  $p < 0.001$ ]. *Post hoc* analysis indicated that latencies to find the platform were significantly reduced on Days 2,3, and 4 of Relearning, as compared to Day 1 of Relearning ( $p < 0.05$ ). Similarly, the rats exposed to DFP between Training and Relearning also appeared to have reduced latencies to find the platform. Although mean latencies for this group were elevated as compared to controls, no significant difference between the groups were evident ( $p = 0.43$ ). Inspection of the latency data for DFP-treated animals revealed that the slightly elevated latency on Days 1-3 of Relearning (relative to controls) are likely attributable to atypically long latencies exhibited by two of the animals on each day of testing. The remaining animals displayed latencies similar to those of control animals.

### **Effect of the DFP Regimen on the Expression of Brain Cholinergic Receptor Subtypes**

#### *Muscarinic (non-specific) Receptor Binding*

For all autoradiographic studies, groups of animals (n=5-6) were culled for brain harvesting on 1, 7 or 21 days after completion of the DFP (250 µg/kg/day/14 days) regimen. The data were expressed as a percentage control (saline injection) means for each group treated with DFP. Table 5 presents autoradiographic data derived from DFP treated and control rats for which brain sections were labeled with, a non-subtype selective muscarinic antagonist. Forebrain regions of relatively high levels (reproducibly greater than background) of [<sup>3</sup>H]QNB binding are listed. Relatively high expression of binding sites were located in cortical, striatal, and hippocampal regions. This pattern reflects primarily the M1 subtype of muscarinic receptor (Wei and Buccafusco, 1994). Despite the robust level of receptor expression in these areas, there was very little change in the number of [<sup>3</sup>H]QNB binding sites in rats treated with DFP. The primary exception occurred in layers 3 and 4 of the parietal cortex, which exhibited a time-dependent loss in binding sites up to 7 days after DFP withdrawal (Table 5). This loss in [<sup>3</sup>H]QNB binding sites, which amounted to about a 23% change from control levels, had returned almost to control levels within 21 days. The results from a typical autoradiographic experiment are shown in Figure 11. Even at one day after DFP withdrawal, there is clear evidence of loss of [<sup>3</sup>H]QNB binding sites within the parietal cortex. This loss in binding sites was observable in all three sections representing rostral through caudal levels of the brain.

#### *Nicotinic (neuronal) Receptor Binding*

Table 6 presents autoradiographic data derived from DFP treated and control rats for which brain sections were labeled with [<sup>3</sup>H]epibatidine, a ligand that preferentially labels high affinity (neuronal) nicotinic receptors. Unlike the distribution of muscarinic receptors [<sup>3</sup>H]epibatidine preferentially labeled sites within thalamic regions, the striatum, the subiculum, and the dentate gyrus of the hippocampus (Fig. 12). Again these results replicate earlier reports for high affinity nicotine binding in the rat brain (Dávila-García et al., 1997). As with the results from the [<sup>3</sup>H]QNB study, there was a very selective loss of [<sup>3</sup>H]epibatidine binding sites, in this case the effect was observed within the dentate gyrus of the hippocampus (Table 6). The loss of nicotinic receptor sites differed in two respects from that observed for the muscarinic sites. First, the loss was more dramatic, with expression declining by almost 50% of control levels. Second, there was only a partial recovery of binding sites by day 21 after DFP withdrawal, with the level still significantly reduced (by about 30%). This reduction in dentate nicotinic receptors is evident in the typical autoradiograph as shown in Figure 13.

#### *Muscarinic (M2) Receptor Binding*

One difference between the neuro-architecture of M1 muscarinic receptors located in the cerebral cortex and nicotinic receptors in the hippocampus is that M1 receptors are considered to exist primarily postsynaptically, whereas nicotinic receptors are largely presynaptic (see, Klein and Löffelholz, 1996). One possibility to be considered was that the effect of DFP was more directed towards presynaptic, rather than postsynaptic cholinergic receptors. To examine this possibility further, we next estimated the relative levels of muscarinic M2 receptors in sections from the same control and DFP treated rats. Like high affinity neuronal nicotinic receptors, the M2

subtype of muscarinic receptors is considered to be located mainly presynaptically in higher brain levels (see Klein and Löffelholz, 1966). For these experiments we used the selective M2 selective muscarinic antagonist [ $^3$ H]AF-DX384. M2 receptor distribution did not always parallel [ $^3$ H]QNB binding sites, suggesting that [ $^3$ H]QNB labeled a different population of receptors than did [ $^3$ H]AF-DX384. For example, M2 receptors were not as highly concentrated in cortical and hippocampal regions (except for the subiculum), rather there were high levels found in the basal ganglia and the superior colliculus (Fig. 14). In contrast to the results with the other two ligands, there was a more pervasive decrease in the expression of [ $^3$ H]AF-DX384 binding sites in the regions examined (Table 7). In sections taken from brains harvested one day after the rats had received the DFP regimen the number of binding sites were significantly reduced in the striatum, dentate gyrus, CA1 hippocampal region, parietal cortex, and subiculum as compared with saline infused control rats. As with the nicotinic receptor decrease, the maximal reduction in M2 receptor expression occurred on withdrawal day 7. By this time only the decreased receptor expression in the striatum, subiculum, and nucleus accumbens was evident. In fact, by withdrawal day 21 the receptor numbers in these two regions were still significantly reduced. The most dramatic reduction in [ $^3$ H]AF-DX384 binding sites was measured in the subiculum where levels declined on day 7 by 64%, with little recovery by withdrawal day 21 (Table 7).

In Figure 15 the DFP induced changes in cholinergic receptor binding sites and acetylcholinesterase levels in cortical and hippocampal regions from 1 to 21 days after DFP withdrawal are compared. One readily apparent feature of the profile of changes in these cholinergic macromolecules is that hippocampal (dentate and subiculum) changes were often greater in magnitude and reduced in recovery compared with cortical changes. Also, those cholinergic macromolecules located mainly presynaptically (M2 and nicotinic) were affected to a greater extent by the DFP treatment than were those (M1 and acetylcholinesterase) that are located largely postsynaptically.

#### *Effect of Pre-training Injection of Nicotine in DFP Treated Rats*

In view of the loss of hippocampal nicotinic receptors produced by the DFP regimen we sought to determine whether nicotine administration post-DFP treatment could reverse the deficit in water maze task acquisition. In this experimental series, we used the standard 14 day saline (1 group) or DFP (3 groups) regimen. Two weeks after completion of the regimen all 4 groups were initiated in the standard water maze task. The saline regimen group received a subcutaneous injection of saline 15 min before the first trial of the first day's session. Likewise, one of the groups that had received the DFP regimen was injected with saline before each day's session. For the other two DFP groups, one group received 0.5 mg/kg of nicotine, and the other received 1.0 mg/kg of nicotine, s.c. before each day's session. As with our earlier experiments, the DFP regimen resulted in impaired performance in the spatial memory task as compared with saline controls (Fig. 16). Even after 4 days of sessions, the DFP treated rats did not approach the proficiency of controls in terms of swim latencies. For those rats that received pre-session injections of nicotine, task acquisition was similar to saline (regimen) controls and task performance was improved compared with the DFP group that received pre-session injections of

saline. The same relationship between the experimental groups were obtained when swimming distance was measured (Fig. 17). Therefore, swim speed (the ratio of swim latency/swim distance) remained constant among the experimental groups.

#### *Effect of nicotine on VACHT mRNA*

VACHT mRNA was detected in both the cerebral cortex and hippocampus in each sample. As previously reported using this probe (Prendergast et al., 1996), no VACHT-PCR product was detected in samples for which reverse transcriptase was omitted, indicating the absence of contaminating genomic DNA. Analysis of the relative peak areas produced by UV detection of cDNA injected onto an anion exchange column indicated that nicotine exposure significantly increased levels of VACHT mRNA (Fig. 18) in the hippocampus [ $F(1,3) = 33.15$ ,  $P < 0.01$ ]. Peak areas for control and (-)-nicotine treated animals averaged  $1.06 \pm 0.02$  and  $1.23 \pm 0.01$  a.u., respectively. In the cerebral cortex, expression of this mRNA was also significantly increased 1 h following (-)-nicotine exposure [ $F(1,8) = 11.7$ ,  $P < 0.01$ ]. Peak areas for control and treated animals averaged  $1.73 \pm 0.42$  and  $3.59 \pm 0.59$  a.u., respectively, in the cortex.

#### *Effect of the Seed Oil of Celastrus Paniculatus (CP) on Spatial Memory Impairment Induced by Scopolamine*

*Celastrus paniculatus* (CP), a medicinal plant from India has been reputed to be useful as a pharmaceutical aid for learning and memory. The substance also has been reported to improve maze performance in rats. The drug is well known in India and has been used by thousands of people over many years to relieve various medical problems without significant side effects. We investigated the effects of the seed oil of CP on the 6 day performance of young adult rats in a navigational memory task - the Morris water maze. Once daily, oral treatment with CP (50 - 400 mg/kg) over 14 days produced no significant effect on body weight compared to the vehicle group. Animals appeared to behave normally in their home cage and test environments. Also, there were no obvious signs of "cholinergic" toxicity (tremor, convulsions, salivation, fasciculations, lacrimation etc.) observed after administration of CP at any dose or regimen.

In the first experimental series, during the 6 days of water maze testing, rats treated chronically with vehicle and administered scopolamine prior to maze testing (S) exhibited significantly longer swim latencies in comparison with either control rats not administered scopolamine (C), or compared with the two groups receiving the chronic CP regimen (CP or CPS) ( $F_{3,20} = 12.39$ ;  $P < 0.0001$ ). Impairment was evident even on the first day of testing in that the (S) group required 85 sec on average to locate the hidden platform. In contrast, the (C), (CP) and (CPS) groups required only 60, 45 and 69 sec, respectively, to locate the platform. Since 4 trials were run per day, it was possible to examine task performance on the very first trial before learning could take place. Under these conditions the 4 experimental groups did not exhibit significantly different swim latencies. For the (C), (S), (CP) and (CPS) groups the mean swim latencies on the very first trial were, respectively,  $78 \pm 13$ ,  $90 \pm 0$ ,  $77 \pm 9$  and  $81 \pm 9$  sec. Chronic daily administration with 200 mg/kg CP for 14 days produced a statistically significant decrease in the

latency to find the platform on first day but no difference was observed on subsequent days. As shown in Figure 19A, chronic treatment with CP significantly reversed the impairment in maze performance produced by scopolamine [(CPS) vs. (S)]. *Post hoc* analysis indicated that significance differences were observed between (CPS) and (S) over first four days of water maze experiment. Swim latencies exhibited by all groups of rats were gradually, but significantly decreased from day 1 to day 6 ( $F_{5,15} = 38.8$ ;  $P < 0.0001$ ). This decrease in swim latencies over the 6 test days indicated that all of the rats eventually learned the task irrespective of drug treatment.

Consistent with the swim latency results, the distances traveled by the (S) group rats were significantly longer than either control rats not administered scopolamine (C), or the other two groups rats that received the chronic CP regimen (CP or CPS) ( $F_{3,20} = 14.98$ ;  $P < 0.0001$ ). With some minor exceptions, the between-group differences obtained for the swim distance data were similar to those obtained for the swim latency data (Fig. 19B). There were no significant between-group differences observed for the animals performing the visual acuity task administered on the seventh day of testing. Group means for this task were 14, 10, 9, and 7 sec for (C), (CP), (S) and (CPS), respectively. The cholinesterase activity of frontal cortex homogenates were not significantly different between (C) and CP groups ( $0.0069 \pm 0.001$  and  $0.0061 \pm 0.0003$   $\mu\text{mol/min}$ , respectively).

For the locomotor activity experiment, all 4 experimental groups received their respective regimens 20 min prior to testing. Horizontal activity (horizontal beam breaks), movement time, and the number of stereotyped movements (repetitive breaking of the same beam) were significantly enhanced by scopolamine. In contrast, vertical activity (vertical beam breaks) was not significantly affected by the muscarinic receptor antagonist (Table 8a). Chronic pre-treatment with CP slightly increased all locomotor activity parameters compared to control but no significant differences were observed. Unlike, its anti-scopolamine effect on water maze performance, the chronic CP regimen did not significantly affect the increase in horizontal motor activity and stereotypy produced by scopolamine (Table 8a). Using the same experimental paradigm described above, a second series of experiments was performed in which two additional doses (chronic) 50 mg/kg and 400 mg/kg of CP were evaluated for their ability to reverse the scopolamine-induced deficits. The data for the second and third days of maze testing are presented in Figure 20 along with those for the 200 mg/kg dose for comparison. As with the 200 mg/kg dose of CP, both the 50 and the 400 mg/kg doses completely reversed the scopolamine deficit in maze performance ( $F_{5,48} = 8.89$ ;  $P < 0.0001$ ). All 3 doses appeared to be maximally effective in this regard, and as such, there was no apparent dose-response relationship over this range of doses. The data suggest that CP exhibits a wide therapeutic window (Fig. 20).

In the second experimental series the rats only received acute injections of drugs or vehicle 20 min prior to maze testing. Under these conditions scopolamine treated rats (S) exhibited significantly longer swim latencies in comparison with either vehicle (C) or (CP) treated rats ( $F_{3,20} = 6.37$ ;  $P < 0.01$ ). Single dose (acute) treatment with CP (200 mg/kg) 20 min prior to testing was not as effective as chronic administration in reversing the scopolamine-induced deficits in

water maze performance. In fact, the learning curve (swim latencies) for the combined CP and scopolamine treatment (CPS) was slightly, but not significantly improved compared with the scopolamine (S) group (Fig. 21A). Also, for acute administration, CP alone (CP) had no effect on swim latencies in comparison with the (C) group. As with the results from the chronic study, no significant differences were obtained among mean swim latencies for the 4 experimental groups on the very first trial of the first day of testing. Essentially identical results were obtained when swim distances were compared among the groups (Fig. 21B). Finally, acute administration of CP alone (CP) had no effect on locomotor activity in comparison with the (C) group. As with the chronic administration regimen, acute CP was completely ineffective in reversing scopolamine-induced increases in locomotor activity (Table 8b).

#### *Effect of co-administration of pyridostigmine bromide on DFP-induced impaired spatial memory*

The purpose of this experiment was to examine the extent to which protracted exposure to pyridostigmine bromide (PB) may enhance the expression of subtle locomotor symptoms and spatial learning impairment upon exposure to DFP. Rats were administered PB (0.40 mg/kg, p.o., t.i.d.) or DFP (250 µg/kg, s.c., once daily), or a combination of both for 7 days. Immediately after treatment with the drugs, the animals were monitored for their subtle locomotor activity and the data recorded. Figure 22 shows that each DFP regimen, particularly the combined PB/DFP regimen, produced reductions in rearing after 1-3 days of administration. Rearing in animals receiving PB or DFP alone began to recover to near-control levels after 5-6 days of exposure whereas those receiving the combined PB/DFP regimen continued to display motor abnormalities throughout the observation period. Next the animals were subjected to water maze testing 1 week after the completion of each drug regimen. As indicated in Figure 23, DFP treated rats were significantly impaired in water maze performance even on the first day of testing as compared with the saline control group. The group that received the combined PB/DFP regimen exhibited near saline levels of maze performance. The group that received PB alone exhibited a similar level of task performance except for the anomalously high mean latency recorded during the third session (day) of testing.

## **DISCUSSION**

The present data are consistent with the development of subtle, but significant impairment in new task learning in rats after being exposed to a chronic low level regimen of the OP DFP. This effect of the DFP regimen was observed in numerous individual studies using different sets of control animals over the course of the entire project. Rats were impaired in task performance from 1 to 21 days after withdrawal from the regimen. Thus this is a reproducible phenomenon as it pertains to rats learning a spatial memory task. In our initial study we sought to determine a dose of DFP that could be administered chronically, but which would not induce overt signs of cholinergic toxicity. However, even with the dose we eventually chose to be used in our standard regimen (250 µg/kg), we observed subtle and reversible changes in locomotor and

sensory behaviors. Both olfactory reactivity and spontaneous locomotor activity were suppressed by DFP beginning 3 days after the start of DFP exposure; but by the end of the regimen treated rats were almost completely indistinguishable from their control-regimen counterparts. The development of behavioral tolerance to OP agents has been well documented (Brodeur and DuBois, 1975; Russell et al. 1975; Costa et al. 1982) and has been suggested to reflect the down regulation of ACh muscarinic (Bushnell et al. 1991; Schwartz and Kellar, 1983; Schiller, 1979; Yang et al. 1993) and nicotinic (Schartz and Kellar, 1983) receptors. Over the course of the study, however, a small percentage of the animals treated with the standard regimen displayed obvious signs of cholinergic toxicity. We did not undertake to determine why a small fraction of the subjects responded vigorously to the 250  $\mu\text{g/kg}$  DFP regimen, whereas most animals did not exhibit the slightest symptom of cholinergic overstimulation. One possibility is that a significant population of albino rats are DFP-sensitive. Indeed, this selective susceptibility to DFP within the general population has been exploited to develop inbred strains of "hypercholinergic" and "hypocholinergic" rats (Shiromani and Overstreet, 1994; Markou et al., 1994). The potential impact of this finding in rats as it pertains to human low-level OP exposure has been largely unexplored.

During these initial dose finding studies, it was of interest to note that a significant increase in body weight was observed with continued administration of the lowest dose regiment (50  $\mu\text{g/kg}$ ) of DFP. By the last day of DFP administration, a mean increase in weight of  $69.70 \pm 2.03$  g above Day 1 weights was observed. Although the specific mechanism contributing to the weight gain to DFP is unclear, it may be related to the concomitant increase in spontaneous locomotor activity. For example, administration of carbachol directly into the hippocampus stimulated feeding in rats, an effect which paralleled a dramatic increase in spontaneous locomotor activity (Grant and Jarrard, 1968). In the present study, an increase in locomotor behavior above control levels was evident throughout the time course of administration of the 50  $\mu\text{g/kg}$  dose, suggesting a stimulatory psychomotor action was produced by the drug. The increase in body weight observed, therefore, may well reflect a hyperphagic response to non-specific excitation of locomotor activity, as has previously been suggested with other hyperphagic drugs (Chauloff et al. 1988; Montgomery et al. 1988).

One consequence of protracted neurotoxicity affecting cholinergic neurons and receptors may be impairment of learning and/or memory processes. Pharmacological blockade of neuronal nicotinic and muscarinic receptors impairs learning and memory processes (see Warburton and Wesnes 1984; Bannon et al., 1995). Moreover, the loss of critical cholinergic neurons and specific receptor subtypes also is associated with cognitive impairment in Alzheimer's disease (Bartus et al. 1985). Because cholinergic receptor density is reduced in brain regions important for the processes of learning and memory after chronic OP exposure, the impairment in navigational task performance by our rats was not unexpected. In the present study, rats which began training in a spatial navigation task 3 days after the last DFP administration were impaired, relative to controls, in their ability to learn the task on each of 4 consecutive days of training. However, performance on Day 4 was only slightly worse than controls. The DFP group demonstrated (as did the control group) an ability to improve in their performance of the



task each day. Thus, the protracted effects from the DFP regimen allowed the rats to learn the task, but with reduced efficiency compared with controls. Impaired task performance was not a consequence of drug effects on locomotor activity or visual acuity. Both swim speed and ability to find a highly visible platform were similar for DFP- and saline-treated animals, suggesting a selective impairment of learning processes.

To further examine the possibility that impaired learning reflected the non-specific peripheral effects of DFP administration, a separate group of rats was administered the quaternary carbamate cholinesterase inhibitor echothiophate iodide in a similar 14-day treatment regimen. The dose of echothiophate was chosen to provide a similar degree of inhibition of plasma cholinesterase as did the 250  $\mu$ g dose of DFP. At no time during training in the water maze was impairment of learning evident. Further, both swim speed and visual acuity were unaffected by echothiophate. As a whole, these findings suggest that peripheral AChE inhibition does not result in impaired learning in the water maze task and that impairment of task performance by DFP is mediated within the CNS.

AChE activity in both the frontal cortex and hippocampus was suppressed to less than 50% of control levels at a time point corresponding to the start of training. At a time point corresponding to the end of testing (7 days after the end of drug administration), cortical enzyme activity had recovered to 83.5% of control levels, representing an increase in activity of greater than 40% in the preceding 4 days. Hippocampal AChE, however, recovered at a much slower rate, to only 64.4% of controls at this time point. This represents an increase in enzyme activity of only 14.4% over this same 4 day period. While the specific reason for the difference in enzyme recovery rates in these regions is unclear, it may be associated with regional differences in the rate of compensatory pseudoesterase induction or the relative rate of formation of DFP-enzyme complex in these different brain regions. At the end of the second time point of water maze testing (21 days after the final DFP administration) both cortical and hippocampal enzyme levels were not statistically different from control levels. Therefore, although the AChE recovery appears to be impaired in the hippocampus relative to the frontal cortex, enzyme activity in both regions recovers to control levels by 21 days after the end of drug treatment. Despite the recovery of AChE, water maze learning was still impaired at this time point (days 17-21) in animals administered DFP (250  $\mu$ g/kg) for 14 days and allowed to remain undisturbed for 16 days thereafter. Therefore, ongoing inhibition of AChE activity in these regions does not appear to account for this protracted impairment of learning. As will become evident in the discussion below, it is more likely that decreases in cholinergic receptor expression are more likely to play a role in mediating the cognitive deficits to the DFP regimen. Nevertheless, given our finding of impaired AChE recovery in the hippocampus, relative to the cortex, the hippocampus may represent a site of significant and protracted OP neurotoxicity. Evidence of a potent effect of DFP on hippocampal EEG activity is consistent with this possibility. Several authors have reported the ability of acute DFP administration to induce focal epileptiform activity in hippocampal neurons which is attenuated by muscarinic blockade (Baker and Kratky, 1968; Baker and Kratky, 1968). Both these findings and those of the present study demonstrate a significant disruption of hippocampal neuronal activity after DFP exposure. Disruption of

hippocampal function has significant implications regarding the effects of OP agents on cognitive function. The hippocampus undoubtedly mediates several different forms of learning including both cellular long-term potentiation (Bohme et al., 1991) and learning of behavioral tasks (Huang and Lee, 1995). With regard to behavioral learning, hippocampal activity appears to be of particular import in the formation of working memory, such as that required to learn an unfamiliar task. Following hippocampal lesions, working memory is more severely disrupted than is reference memory of well-learned tasks (Oltan and Papas, 1979; McLamb et al., 1988). Further, DFP has previously been reported to disrupt performance on novel, but not familiar, cognitive tasks (Upchurch and Wehner, 1987). Bushnell and colleagues (1991) demonstrated the ability of chronic DFP administration to impair performance on a well-learned delayed matching task in rats during drug administration. However, following drug withdrawal, performance of this task returned to pre-drug control levels. These data are consistent, therefore, with the suggestion that chronic, low-level DFP administration may produce protracted impairment of hippocampal processes which selectively mediate working memory.

Monkeys treated chronically with DFP were not impaired in their ability to perform a well learned DMTS task, or in their response to distracting stimuli. This finding was quite unexpected given our results with the rats in the spatial memory task. Even after the appearance of severe cholinergic toxicity and a marked reduction in the activity of erythrocyte cholinesterase activity, the monkeys returned rapidly to baseline levels of task performance once the drug regimen was discontinued. The lack of a continued disruptive effect of DFP on DMTS performance may be related to the extensive familiarity of our monkeys with the task. Each monkey had been performing the task for a minimum of 1 year prior to the start of DFP administration. The effect of DFP exposure on reference memory-related tasks, therefore, may depend upon the degree of task familiarity and/or the cognitive capabilities of the animal being test (e.g. rat vs. monkey). Tasks most affected by DFP exposure in primates may be limited to those that possess significant novelty. To suggest this possibility are our results in which rats were examined in either the DSDT or the relearning version of the water maze task, wherein those subjects that received the DFP regimen performed at levels of accuracy similar to those of controls. Thus development of reference concepts (by prior training) of delayed matching, discrimination, and spatial navigation strategies appears to offer resistance to disruption by cholinesterase inhibition. It is interesting to note that the relative amount of training in the two different rodent paradigms employed in this study were markedly different. Water maze training consisted only of 4 consecutive days (comprised of 4 trials each day) of exposure to the task whereas as DSDT training consisted of 14 weeks of training with a 5 sec delay interval. Therefore, the relative amount of training (or over-learning) does not appear to be a significant variable influencing response to the DFP treatment regimen. Only a relatively brief exposure to a task appears to be sufficient for the development of a reference construct.

The resistance of performance of these delayed recall tasks to impairment (independent of toxicity) following exposure to DFP is almost certainly associated with the tasks' familiarity to the animals. Previous studies in rodents suggest that novel tasks (those most reliant on working

memory) are more severely impaired by DFP than are familiar tasks which rely primarily on reference memory. Upchurch and Wehner (30) reported that spatial learning in mice was impaired by chronic DFP administration only if training was initiated after exposure to DFP. Performance of this task was not disrupted by chronic DFP exposure if animals had been trained prior to DFP exposure. Work conducted in this laboratory has shown that spatial navigation learning of a novel task in rats is impaired for up to 20 days following withdrawal from chronic exposure to a sub-toxic dose of DFP (0.25 mg/kg; 26). In contrast, Bushnell and colleagues (10) report that accuracy on a well-learned matching-to-position task in rats was impaired during chronic DFP administration (0.2 mg/kg), but that performance quickly returned to baseline, pre-drug levels soon after DFP withdrawal. In the present study, each monkey was well-trained in the DMTS task having performed it five days each week for a period of at least 3 years. Although working memory of sample stimulus characteristics was a necessary component of accurate recall, complex learning of a novel task was not required.

Performance of the tasks employed in this study were, therefore, largely dependent on reference memory of the delayed matching and discrimination, as well as spatial navigation, concepts. These tasks may be described as "mixed memory" tasks, which have previously been suggested to be resistant to disruption following OP exposure. This may suggest then, that extended exposure to low levels of OP agents can produce selective impairment of working or short-term memory, but may not significantly affect long-term, reference memory.

Three tritiated cholinergic receptor ligands were used to assess the presence of receptors in brain sections taken from rats exposed to our chronic DFP regimen or control rats: QNB (non-selective muscarinic), AFDX 384 (M2 selective), and epibatidine (non-selective nicotinic). QNB measured primarily M1 receptors since this subtype is expressed to the greatest extent in forebrain regions. Epibatidine measured all 3 major subtypes of nicotinic receptors:  $\alpha 4/\beta 2$ ,  $\alpha 3/\beta 4$ , and  $\alpha 7$ . From these data it can be ascertained that (1) cholinergic proteins that are largely expressed post-synaptically (AChE and M1) were either least affected (M1) or had more rapidly returned to control levels (AChE) than were those proteins that are primarily expressed in presynaptic nerve endings (nicotinic and M2); and (2) hippocampal cholinergic proteins were more affected than cortical proteins. The mechanism of selectivity for the effects of the DFP regimen on hippocampal neurons as compared with cortical neurons (and compared with other brain regions not illustrated here) is not yet apparent. However, this selectivity fits well with the performance deficit in water maze performance, which is known to require intact hippocampal processing. In fact, we would have been surprised if the neurochemical effects to the OP treatment were not anatomically selective in view of the fact that our rats were not impaired in all types of memory tasks. Parenthetically, the same may be said for what is known regarding victims of chronic OP intoxication and Persian Gulf War Illness. Of particular relevance to the proposed study is the observation that presynaptic cholinergic receptors appear to be singularly susceptible to the toxin as compared to postsynaptic receptors. This scenario is not unlike that which is known to occur in Alzheimer's disease, wherein nicotinic and M2 receptors residing on the endings of cholinergic basal forebrain and medial septal neurons become depleted. The target tissue densities of postsynaptic cortical and hippocampal M1 receptors are generally preserved.

Thus, our findings are in keeping with the possibility that OP agents may directly or indirectly inactivate axonal transport of these macromolecules from cell bodies in the medial septum to sites within the hippocampus.

Central nicotinic receptors have been studied as potential pharmacologic targets to improve learning and memory in experimental animals (Levin, 1992; Arneric and Williams, 1993; Arneric et al., 1995). The impetus for such research stems partly from the observation that central nicotinic receptors become depleted in patients dying of Alzheimer's disease (Quirion et al., 1986; Whitehouse et al., 1986; Flynn and Mash, 1986; Nordberg and Winblad, 1986; Schroder et al., 1991). Previous studies from this laboratory have demonstrated that nicotine improves short term memory performance in both young and aged monkeys (Elrod et al., 1988; Buccafusco and Jackson, 1991). Using doses of the drug similar to those we employed in monkeys, nicotine was demonstrated to enhance cognitive performance in Alzheimer's patients (Newhouse et al., 1988; Sahakian, et al., 1989). The involvement of nicotinic neurotransmission in cognitive function is further substantiated by observed deficits in cognitive performance following administration of mecamylamine, a nicotinic receptor channel blocker, to rodents (Levin, 1992), monkeys (Elrod et al., 1988), and humans (Newhouse et al., 1992). Therefore, in the next series, we evaluated the ability of nicotine administered to rats each day prior to water maze testing to reverse the DFP-induced inhibition of task performance. Using the same 2 week DFP regimen, we tested control and toxin treated rats beginning 2 weeks after the regimen was discontinued. DFP-treated rats exhibited a significantly reduced rate of learning compared with control rats. In the DFP groups that received one of two doses of nicotine each day just before maze testing, the rate of learning was similar to that for controls. In fact, the DFP groups that received nicotine exhibited enhanced performance even relative to the control (non-DFP) group on the first day of testing. While nicotine is used clinically only for smoking cessation, several pharmaceutical companies are currently evaluating novel analogs that are purported to exhibit a reduced side-effect profile relative to nicotine. These compounds soon may be available for treating cognitive deficits associated with long-term OP exposure.

The ability of nicotine to improve memory-related task performance has been shown to outlast the presence of the drug in the plasma and brain (see, Buccafusco et al., 1996). The mechanism for this protracted beneficial effect of nicotine (and certain other nicotinic analogs) is not known. One possibility is that acute nicotine administration sets into motion cellular events that lead to the protracted improvement in task performance. Nicotine-induced neurotransmitter release may be initiated by alterations in the expression of genes associated with transmitter synthesis. Acute nicotine administration has been reported to increase expression of mRNA species encoding the catecholamine synthetic enzymes tyrosine hydroxylase and dopamine  $\beta$ -hydroxylase in both rat adrenals and in pheochromocytoma cultured cells (Hiremagalur et al. 1993; Koistinaho et al. 1993; Hiremagalu and Sabban, 1995). In addition, *in vivo* injection of nicotine (0.8 mg/kg) also produced increases in mRNA for tyrosine hydroxylase in the locus coeruleus (Mitchell et al., 1993). These changes were correlated with increased enzyme activity and catecholamine release in each model system. In this study we determined whether acute nicotine injection could increase the levels of the mRNA that encodes the vesicular acetylcholine transporter, a marker

for cholinergic nerve terminals, and an important component for transmitter synthesis and release. The present data provide further evidence that acute stimulation of nAChRs influence neuronal gene expression systems. VACHT mRNA is present in the hippocampus, indicating that cholinergic soma are likely to be present in this brain region and, as efferent cholinergic fibers are not thought to originate in the hippocampus, these cholinergic soma may be short projection interneurons. In both the cerebral cortex and the hippocampus, nicotine administration increased the amount VACHT RT-PCR product detected. VACHT mRNA has previously been reported to be increased by up to 3-fold in cultured murine septal cells incubated with retinoids, neurotrophic factors, and cAMP (Berse and Blusztajn 1995). In the present study, VACHT mRNA was increased above control levels by 15.7 and 65.2 %, respectively, in the hippocampus and cortex.

The sensitivity of VACHT mRNA expression to nAChR activity may be associated with the induction of gene expression systems such as that mediated by CREB. In this regard, acute administration of nicotine has been reported to increase the phosphorylation of CREB (Hiremagalu and Sabban 1995). Thus, upon activation of gene expression in CREB-sensitive genes, G3PDH may be increased to facilitate nuclear tRNA export as has previously been reported (Sirover 1997). VACHT expression may be initiated after presynaptic ACh release and subsequent ACh synthesis in response to this release. Phosphorylation of CREB is initiated by a rise in intracellular cAMP and subsequent A-kinase activation (Della Fazia et al., 1997). This may suggest that the promoter regions of the VACHT gene contains the cAMP response element (CRE), that when bound by CREB, initiates gene expression. Regarding the VACHT, this view is supported by evidence that the entire coding sequence for the VACHT is contained within the first intron of the gene encoding the ACh synthetic enzyme choline acetyltransferase (ChAT) and that the ChAT promoter region does contain the CRE (Erickson et al., 1994). Not surprising, cAMP incubation also increases expression of ChAT mRNA in these same septal cells (Berse and Blusztajn, 1995). Significantly, the cAMP-induced increase in ChAT and VACHT mRNA in these cells was associated with increased levels of intracellular ACh. It may be likely that increased VACHT mRNA observed in the present study was also associated with an increase in ACh in these regions, though this was not measured. It remains to be determined if this nicotine induced cascade of ChAT expression, ACh synthesis, and VACHT expression persists for a significant period of time after nAChR stimulation and if this is associated with persisting presynaptic release of ACh. Evidence of such a persisting effect may aid in explaining the protracted beneficial cognitive effects of nAChR stimulation (Levin et al., 1990; Terry et al., 1993; Prendergast et al., 1997).

Since muscarinic M2 receptors also were decreased after the chronic DFP regimen we also sought to determine whether it might be possible to address the potential effects of the loss of these receptors by pharmacologic means. Blockade of central muscarinic receptors produces a short-term amnesic response in a wide variety of animal models performing a variety of tasks challenging learning and recall (Elrod and Buccafusco, 1988; Flood and Cherkin, 1981; Hearst, 1959; Levin, 1988). Scopolamine is particularly effective when administered just before task training (Elrod and Buccafusco, 1988). Although the effects of scopolamine on maze

performance and locomotor activity can be reversed by the administration of classical (centrally-acting) muscarinic receptor agonists, particularly inhibitors of acetylcholinesterase (Shannon and Peters, 1990) we were reluctant to use a potential treatment that was similar to the original (DFP) insult.

Traditional or folk medicines have been widely employed for centuries, and they remain one important source for the discovery of new bio-active compounds. Ayurveda, an ancient traditional system of medicine that has been practiced in India since 200 B.C. employs a large number of medicinal plants used in the prevention and treatment of a wide number of diseases. One of these includes the plant *Celastrus paniculatus* Willd. (CP), a plant known for centuries as "the elixir of life". According to Ayurveda, depending upon the dose regimen, CP may be employed as a stimulant-nerve tonic, rejuvenant, sedative, tranquilizer and diuretic. It is also used in the treatment of leprosy, leucoderma, rheumatism, gout, paralysis and asthma. Most of the claims for this plant have not been substantiated in rigorous scientific settings. This includes the purported property of CP germane to this study - its ability to stimulate the intellect and sharpen the memory. The indigenous peoples that use CP know this preparation as a general cognitive enhancing agent. Several reports are now available to support this later notion in the laboratory setting (Karanth et al., 1980; 1981; Nalini et al., 1986; 1995).

The results of the present study confirm the results of the previous studies mentioned above in that they are consistent with the ability of chronic CP administration to enhance the performance of subjects engaged in memory-related tasks. Chronic treatment with 200 mg/kg of CP alone resulted in a small degree of enhancement in water maze acquisition. A marked degree of task enhancement was not expected since the rats were young and presumably cognitively unimpaired. However, even the 50 mg/kg/day dose regimen was effective in improving maze performance when performance was impaired by central muscarinic receptor blockade with scopolamine. The selectivity of CP in affecting the cognitive aspects of this task was suggested by the ability of the drug to reverse the scopolamine-induced impairment of maze performance, but not the scopolamine-induced increase in locomotor activity. Neither scopolamine nor CP regimens affected the level of performance of the first trial of the maze task; one that presumably does not result from learning. It is not likely, therefore, that the drug treatments, altered task performance by affecting non-mnemonic aspects of water maze learning.

Chronic treatment with CP reversed the scopolamine-induced deficits in maze performance, whereas the acute treatment with CP was much less effective in this regard. This finding is consistent with those of the more recent pharmacological studies employing CP. For example, chronic administration of the drug from 3 to as long as 45 days has been demonstrated to be required for maximal drug responses (Bidwai et al., 1987; Karanth et al., 1980; Nalini et al., 1995). However, we cannot at this time rule out the possibility that the lack of an anti-scopolamine action to acute CP administration is related to the fact that in the acute paradigm the rats were not handled or administered solutions orally over 15 days as they were in the chronic study. It is yet to be proven, but perhaps more likely, that CP induces some slowly adaptive cellular change within the CNS. In this study, all three doses 50, 200 and 400 mg/kg/day of CP

reversed the scopolamine deficits but no dose-response relationship was observed. Possibly the range of doses used in this experiment was too narrow to exhibit a dose-response. Clearly, the 50 mg/kg regimen produced a maximal scopolamine reversal. Incidentally, chronic CP administration was associated with no observable side effects in the animals, even with the 400 mg/kg dose regimen.

The mechanism of action by which CP enhances learning and memory performance in behavioral tasks, and by which it reverses scopolamine-induced learning deficits is as yet unknown. CP did not appear to inhibit brain cholinesterase activity, nor were there any symptoms of cholinergic receptor stimulation exhibited by the animals at any time during treatment. Moreover, if CP were acting as a cholinergic agonist, we would have expected to obtain a reversal of scopolamine's amnestic actions after the acute administration of the material. In the present experiment, CP antagonized scopolamine's actions in the water maze but not in the locomotor activity task. Similarly, 5-HT<sub>3</sub> antagonists have been reported to reverse the scopolamine-induced deficits in learning and memory models but not the motor activity (Brambilla et al., 1993). Moreover, 5-HT related drugs often exhibit a significant delay in the onset of clinical benefit, also reminiscent of the effects of CP. On the basis of the results from previous studies and those of the present work, it is possible that one or more of the constituents of CP oil may possess serotonergic receptor antagonistic properties. Further experiments will be necessary to confirm this possibility. If CP can enhance learning and memory in humans, this plant seed oil may be more effective in individuals who are cognitively impaired as a result of chemical or organic brain damage as compared with normal subjects. In the least, these data may provide the impetus for further study of the material, and isolation of its active components.

In the final experimental series, we sought to determine whether concomitant administration of pyridostigmine bromide (PB) could alter the responses to the standard DFP regimen. PB was administered concomitantly with DFP because PB is used by our armed forces as a prophylactic measure against chemical nerve agents. Also, there has been some discussion that PB might have "sensitized" or predisposed to some of the symptoms of Persian Gulf Illness. These experiments should be considered preliminary since we used only one dose of PB (0.40 mg/kg, p.o., t.i.d.), although this regimen was constructed to mimic field doses during the PGW (30 mg oral PB every 8 hr for 1 - 7 days) (Keeler et al., 1991). Both PB alone, and DFP alone caused a reversible inhibition of olfactory behavior over the one week administration period. When the two agents were combined there was a significant enhancement of the inhibition of this behavior. This was perhaps not too surprising, since both compounds are cholinesterase inhibitors. Although PB does not produce irreversible inhibition of the enzyme, the three/day dosing schedule was most likely as effective as DFP at maintaining cholinesterase inhibition. What was surprising, however, was that despite this apparent initial toxic synergism, that PB/DFP treated animals were almost as efficient as saline controls in learning the water maze task. Thus it appears, that under the conditions of these experiments, that prophylactic administration of PB may not only provide protection against nerve agent exposure acutely, but may also help mitigate the cognitive impairment observed some time after toxicant exposure.

## STUDY CONCLUSIONS

1. Low level chronic exposure to an OP agent DFP produces a protracted impairment in the learning of a new task; whereas performance in well-learned tasks are more resistant to the effects of DFP.
2. The cognitive impairment observed after DFP withdrawal was correlated to a protracted decrease in the expression of cholinergic receptors and cholinesterase in very specific brain regions, particularly the hippocampus. Cholinergic macromolecules that are located predominantly presynaptically (nicotinic and M2 muscarinic receptors) were affected to a much greater extent and for a longer period of time than were cholinergic macromolecules having a predominantly postsynaptic location (M1 receptors and cholinesterase).
3. Pre-testing administration of low doses of nicotine completely reversed the DFP-induced impairment in memory-related task performance. This beneficial action of nicotine may be due, in part, to its ability to stimulate gene expression in the synthesis of presynaptic cholinergic markers (e.g., the vesicular acetylcholine transporter).
4. *Celastrus paniculatus* (CP), a medicinal plant from India when administered chronically, selectively reversed the impairment in spatial memory produced by acute central muscarinic receptor blockade, supporting the possibility that one or more constituents of the oil may offer cognitive enhancing properties. Chronic treatment with CP did not alter brain acetylcholinesterase levels and no signs of cholinergic overstimulation were ever noted during or after treatment. Thus, the seed oil of CP may be effective in individuals who are cognitively impaired as a result of chemical or organic brain damage.
5. Prophylaxis with pyridostigmine bromide (PB) initially enhanced the subtle behavioral effects to DFP, but when both drugs were withdrawal, PB-treated animals exhibited near normal levels of memory-related task performance. Thus, PB may not only provide protection against nerve agent exposure acutely, but may also help mitigate the cognitive impairment observed some time after toxicant exposure.



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Figure 1. Olfactory reactivity in animals exposed to DFP (50, 250, or 500 µg/kg) daily for 14 consecutive days. Reactivity was initially suppressed by the two highest doses with behavioral tolerance to the 250 µg/kg dose developing by Day 7. Data represented as mean frequency of rearing and sniffing ± s.e.m. \* =  $p < 0.05$  vs controls; # =  $p < 0.01$  vs controls.



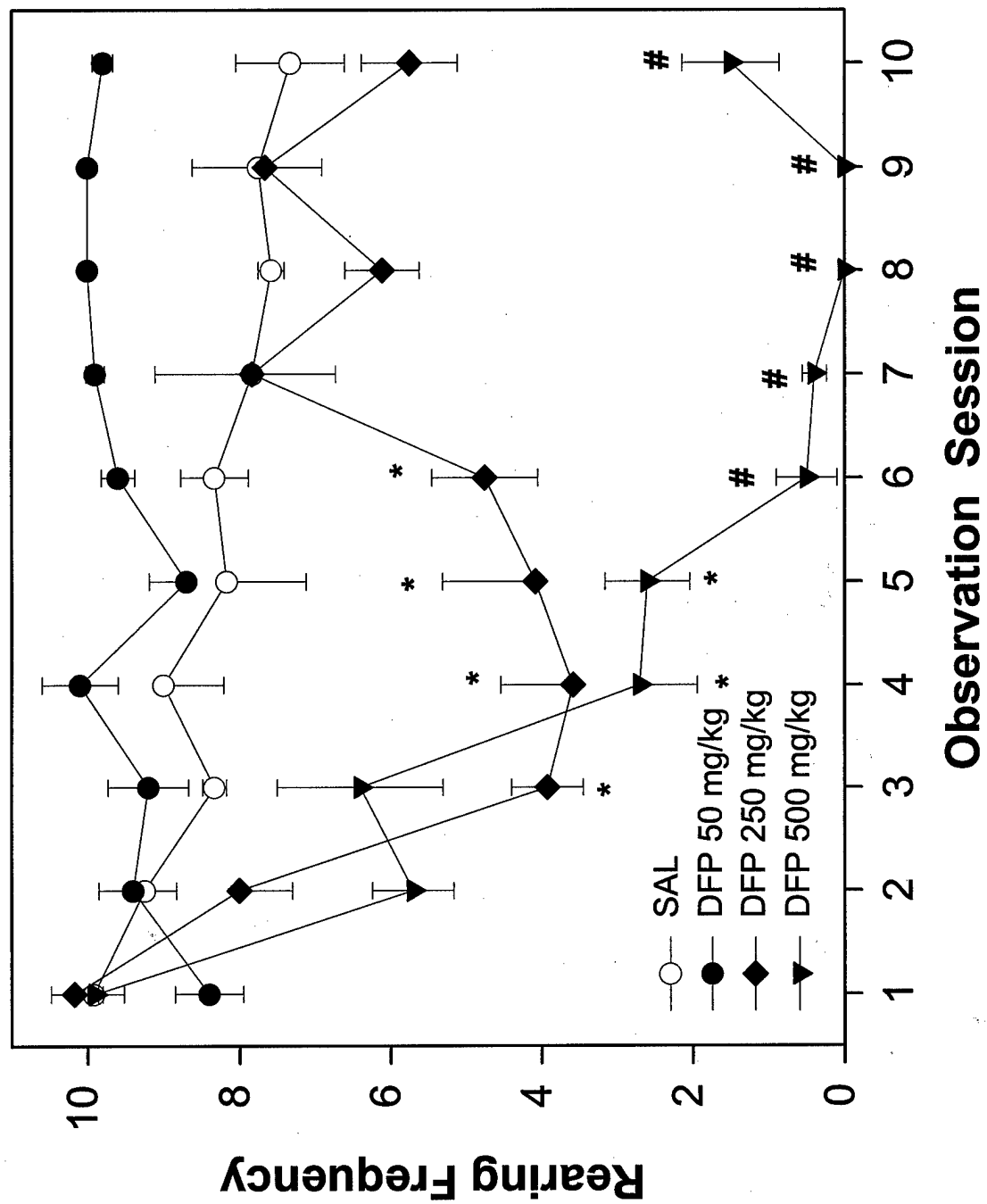


Figure 2. Spontaneous locomotor activity in animals exposed to DFP (50, 250, or 500  $\mu\text{g/kg}$ ) daily for 14 consecutive days. Activity was suppressed by administration of highest two doses with tolerance to the 250  $\mu\text{g/kg}$  dose evident by Day 5. Data represented as the mean frequency of midline crosses  $\pm$  s.e.m. \* =  $p < 0.05$  vs controls; # =  $p < 0.01$  vs controls.

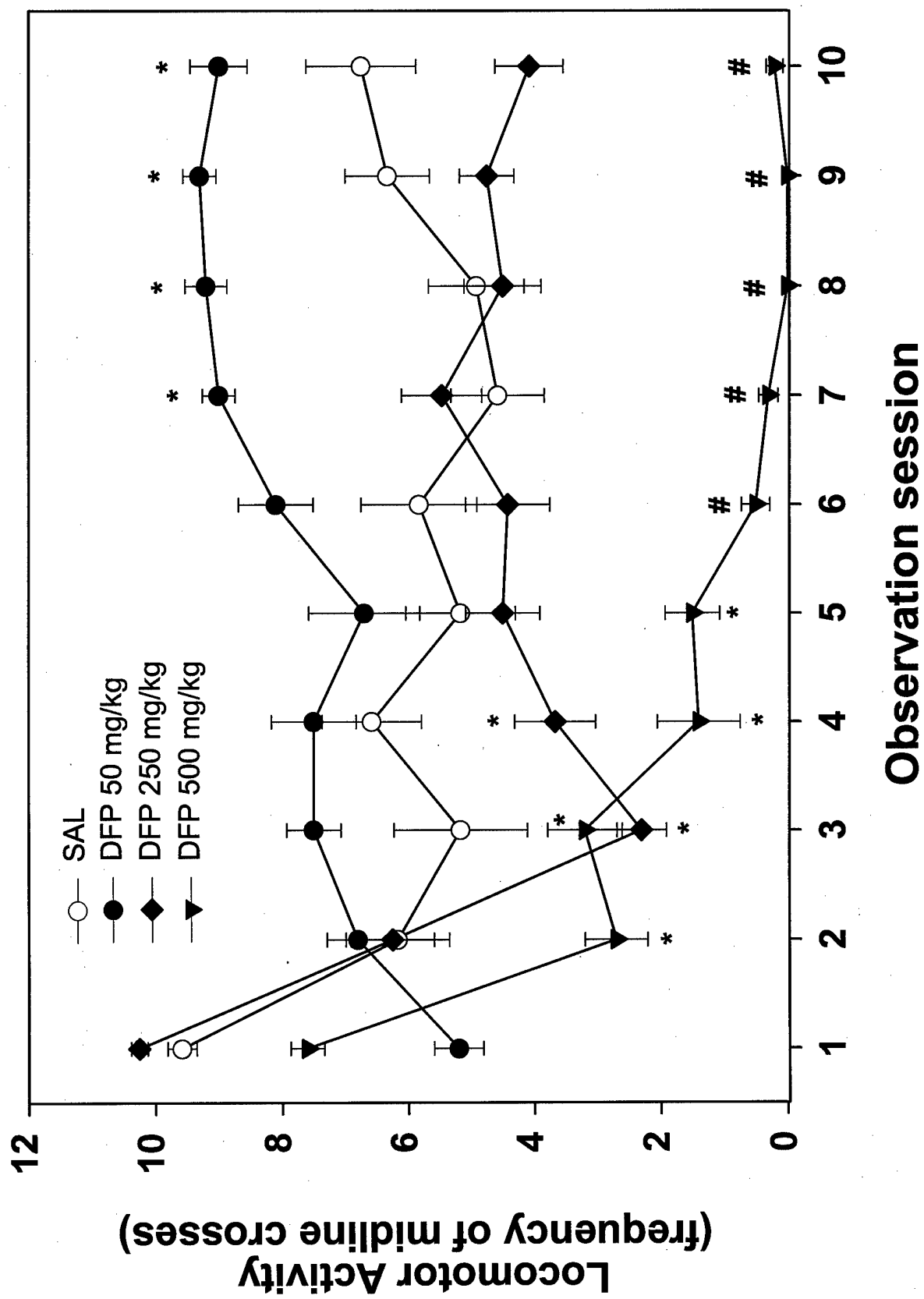


Figure 3. Change in body weight over 14 days of exposure to DFP (50, 250, or 500 µg/kg). Toxicity of the 500 µg/kg dose resulted in significant anorexia and subsequent weight loss. Administration of 50 µg/kg dose induced a steady gain in body weight at a rate above control levels from Day 7 to the end of testing. Data represented as the mean change (in g) in body weight from Day 1 of drug administration. \* =  $p < 0.05$  vs controls; # =  $p < 0.01$  vs controls.

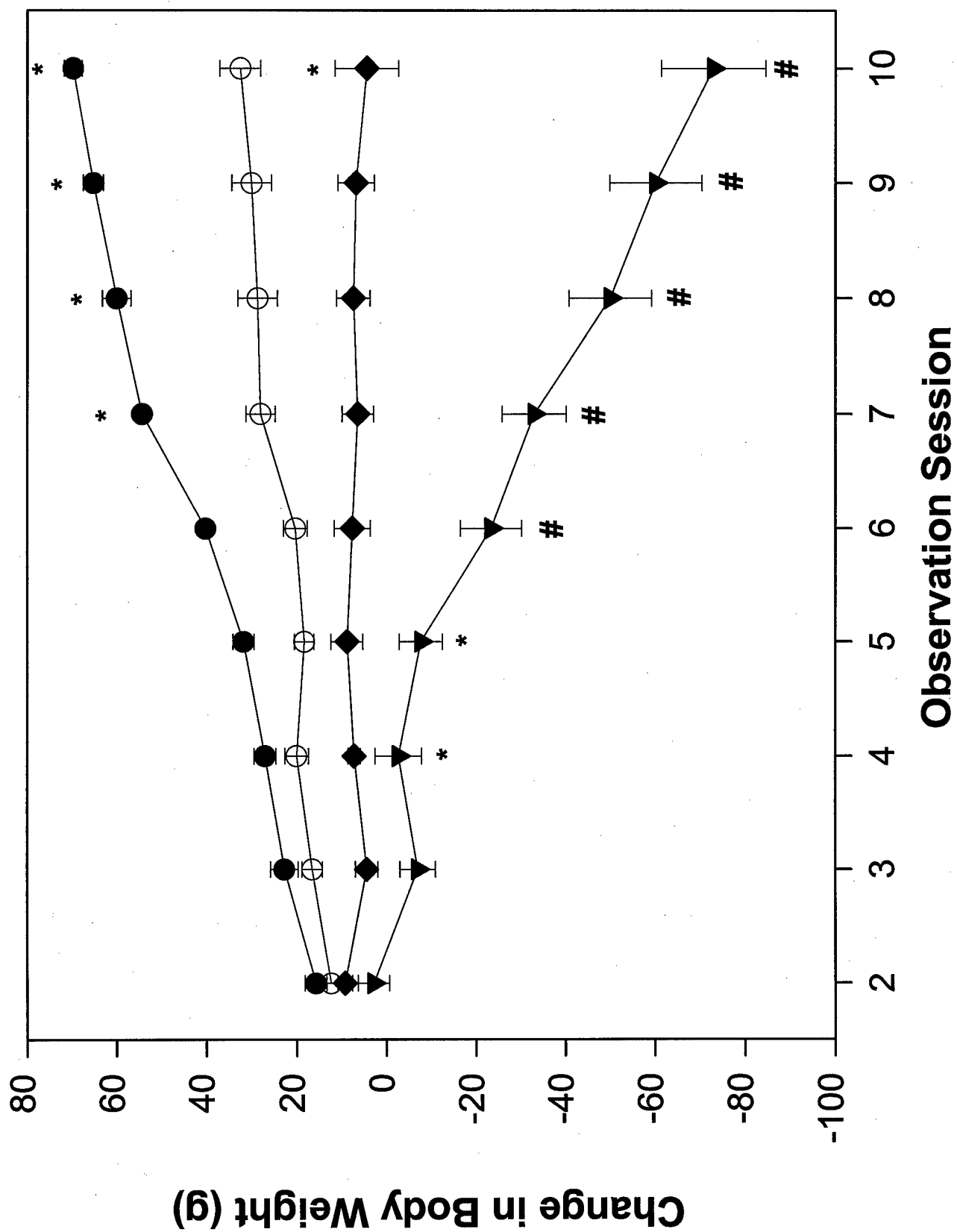


Figure 4. Latencies to find a hidden platform for animals exposed to DFP (50 or 250  $\mu\text{g/kg}$ ) or echothiophate iodide (2.5  $\mu\text{g/kg}$ ) daily for 14 consecutive days. Training was initiated 2 days after the last DFP administration. Animals which received a 500  $\mu\text{g/kg}$  dose of DFP were not tested in the water maze because of the presence of prominent muscle fasciculations, lethargy, and anorexia. Latencies were significantly elevated for those which received the 250  $\mu\text{g/kg}$  dose on Days 1-3. By Day 4, all groups displayed similar latencies to find the platform. Inset: Ability to learn the task was not altered by echothiophate administration. \* =  $p < 0.05$  vs controls.

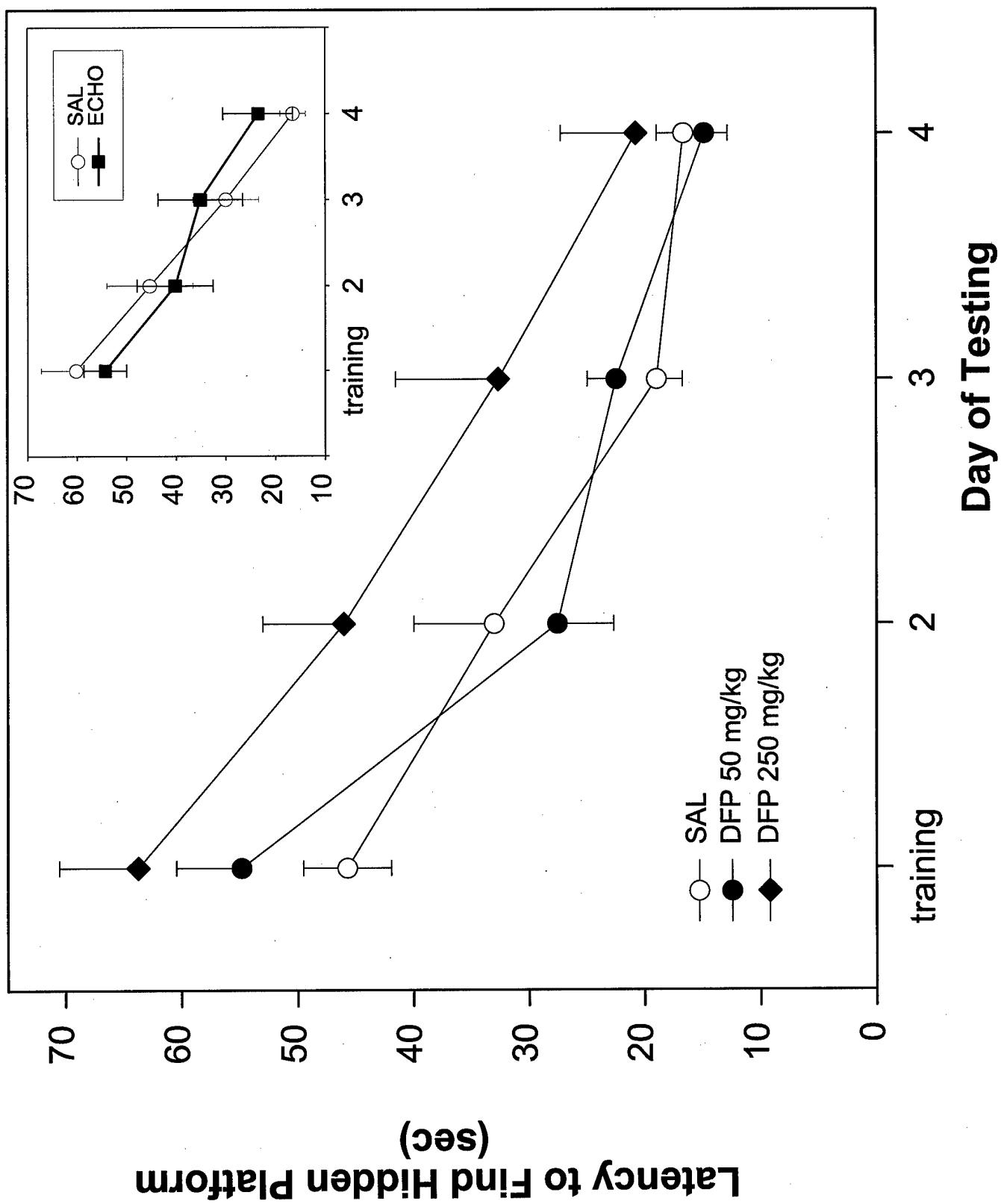


Table 1. Effects of DFP (250  $\mu\text{g/kg}$ ) on swim speed, spatial bias, and visual acuity in the water maze task.

Treatment	Swim Speed (cm/sec)	Spatial Bias (dwell time in sec)	Visual Acuity (latency in sec)
Saline	$23.7 \pm 0.72$	$41.6 \pm 2.35$	$22.3 \pm 3.86$
DFP	$21.6 \pm 1.07$	$40.7 \pm 1.66$	$24.4 \pm 6.90$



Figure 5. Latencies to find a hidden platform for animals exposed to DFP (250 µg/kg) daily for 14 consecutive days. Training was initiated 3 days after the last administration of DFP. Latencies were significantly elevated, as compared to controls, on each day of water maze testing. \* =  $p < 0.05$  vs controls.

### 3 Days After DFP Withdrawal

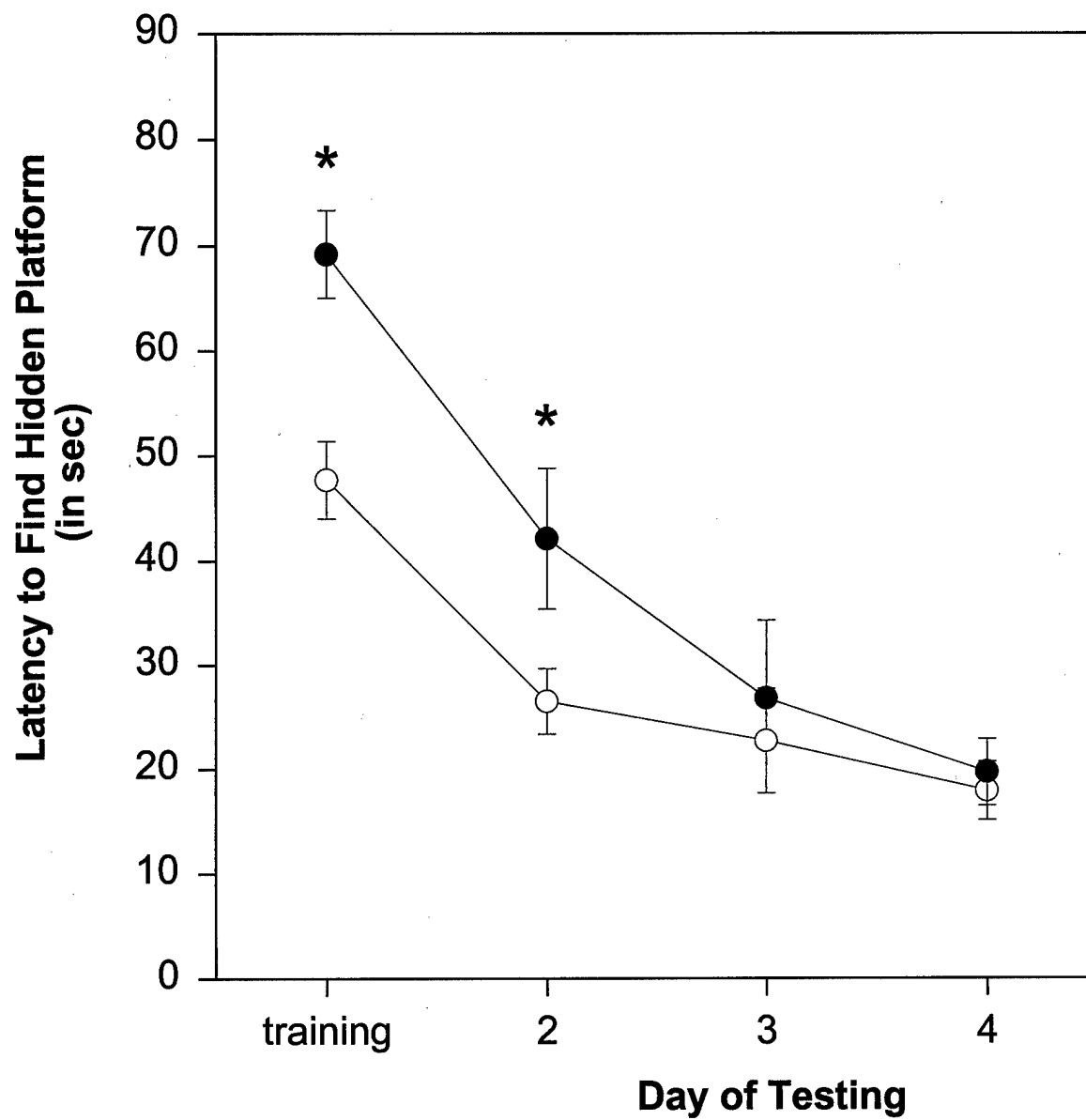


Figure 6. Latencies to find a hidden platform for animals exposed to DFP (250  $\mu\text{g/kg}$ ) daily for 14 consecutive days. Training was initiated 17 days after the last administration of DFP. There was a main "drug effect" (independent of session) by ANOVA [ $F(1,36) = 4.67, p < 0.05$ ]. There also was a main "session effect", [ $F(3,36) = 10.19, p < 0.001$ ]. Figure 7. DMTS accuracy for all delay intervals during 70 days of DFP treatment in 3 young rhesus monkeys. Performance on all intervals, as well as overall performance, was impaired soon after the start of administration of the 20 $\mu\text{g/kg}$  dose of DFP. However, this was accompanied by significant peripheral drug toxicity. Performance returned to baseline levels upon drug withdrawal. Data represented as epochs of 5 consecutive days averaged for each delay (mean  $\pm$  s.e.m.).

### 17 Days After DFP Withdrawal

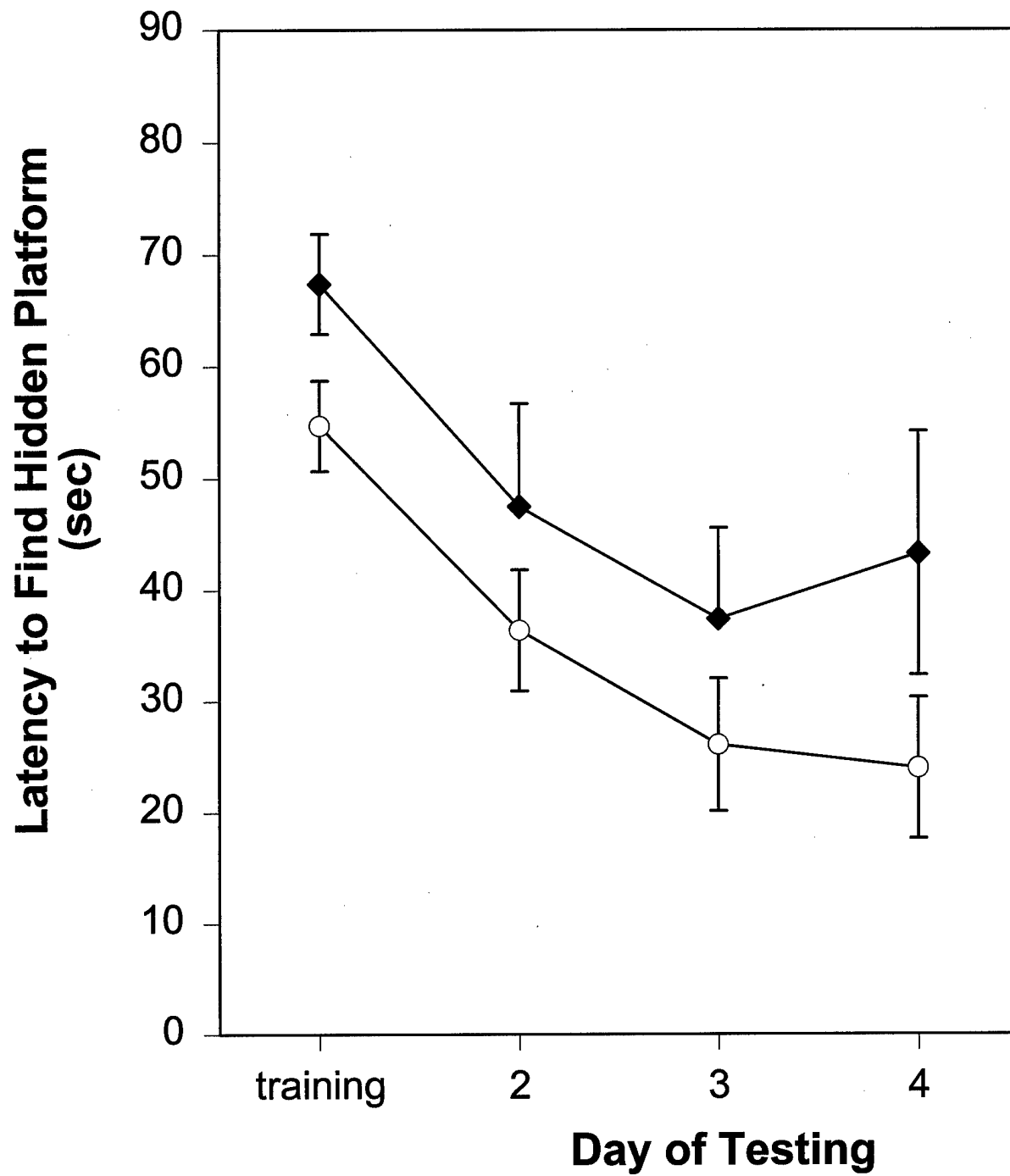


Table 2. Effects of DFP (250  $\mu\text{g/kg}$ ) on AChE activity in the frontal cortex and hippocampus 3, 7, and 21 days after completion of a 14 day treatment regimen. Data expressed as  $\mu\text{mol}$  substrate hydrolyzed/min/mg protein  $\pm$  s.e.m.

Region	3 Days	7 Days	21 Days
Frontal Cortex			
Saline	$0.364 \pm 0.014$	$0.364 \pm 0.035$	$0.319 \pm 0.022$
DFP	$0.155 \pm 0.003^{**}$	$0.298 \pm 0.027^{*}$	$0.291 \pm 0.042$
% of Control	42.58	81.87	91.22
Hippocampus			
Saline	$0.427 \pm 0.012$	$0.469 \pm 0.020$	$0.439 \pm 0.042$
DFP	$0.215 \pm 0.026^{**}$	$0.303 \pm 0.071^{*}$	$0.410 \pm 0.019$
% of Control	50.35	64.61	93.39

\* =  $p < 0.05$  vs saline-controls; \*\* =  $p < 0.001$  vs saline-controls.

Figure 7. Performance of 3 mature monkeys on DMTS trials with zero, short, medium, and long delays intervals across 45 consecutive test sessions during which DFP administration was titrated from 0.01 mg/kg (25 days) to 0.015 mg/kg (15 days) and finally, 0.02 mg/kg (5 days). "End DFP" indicates the point at which monkeys were withdrawn from DFP administration.

# DMTS PERFORMANCE (% CORRECT)

5 DAY PERIOD

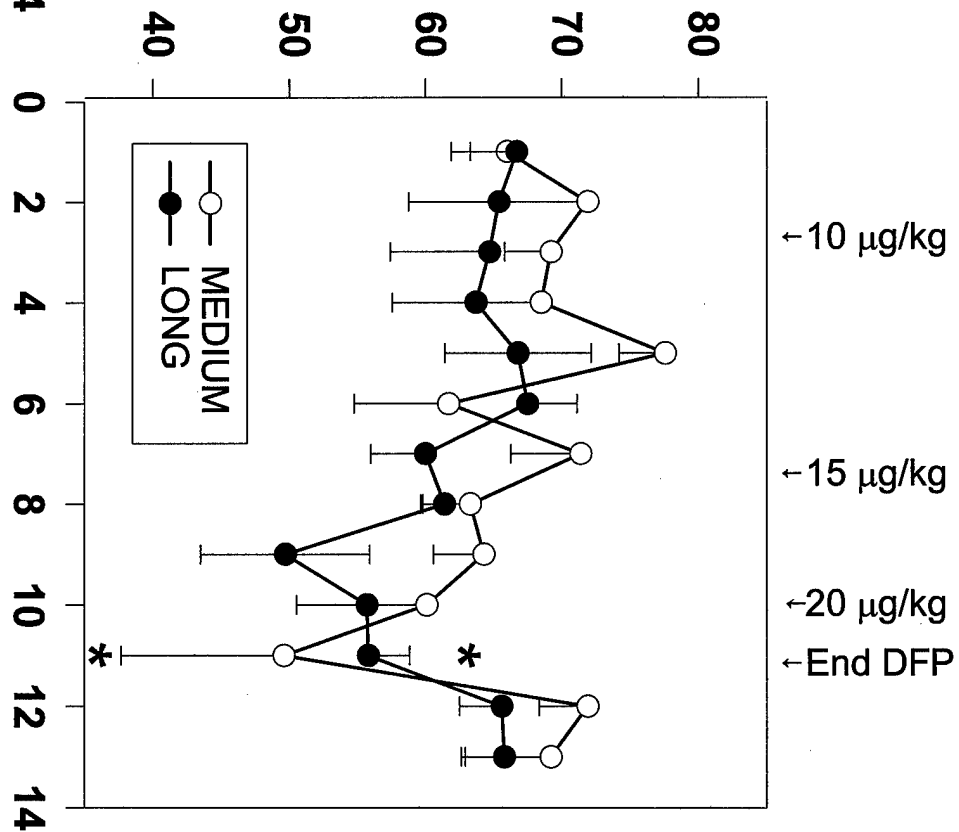
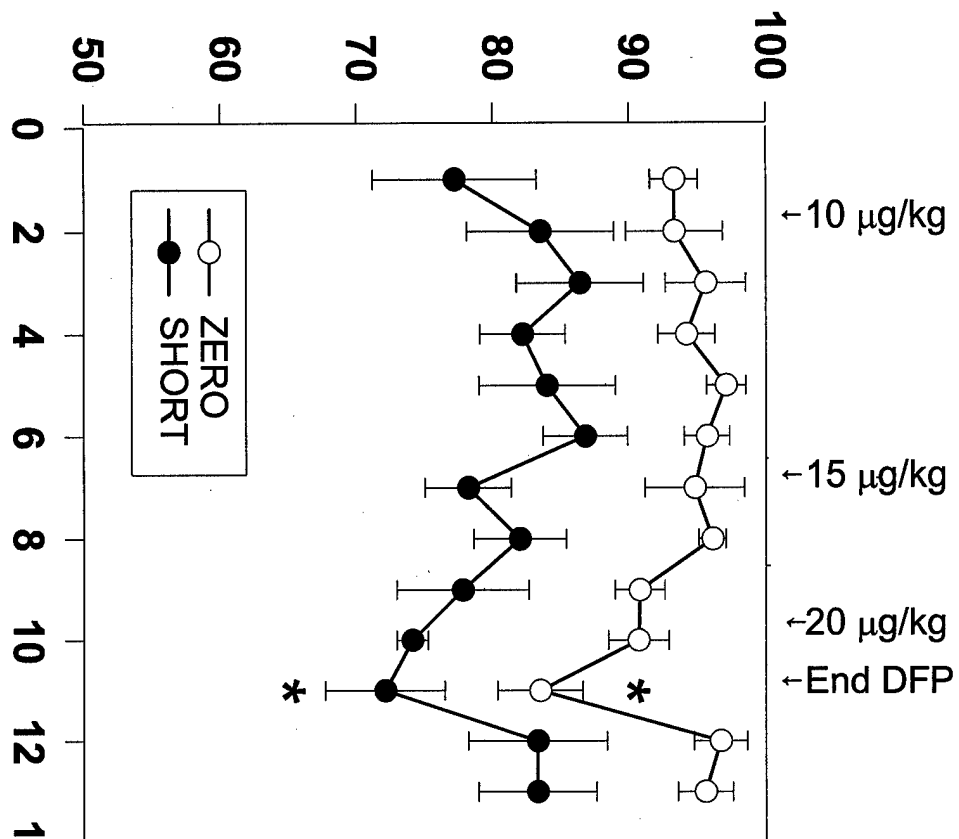


Table 3. Latencies to respond to sample and choice stimuli on correct and incorrect trials during administration of saline or DFP (0.01, 0.015, or 0.02 mg/kg).

Treatment (mg/kg)	Sample Latency (sec)		Choice Latency (sec)	
	correct	incorrect	correct	incorrect
saline	1.85 ± 0.49	1.27 ± 0.15	2.83 ± 0.45	4.98 ± 0.59*
0.01	1.79 ± 0.25	1.75 ± 0.36	2.70 ± 0.53	4.20 ± 0.67*
0.015	3.03 ± 1.39	1.77 ± 0.11	3.19 ± 0.67	5.00 ± 0.83*
0.02	1.32 ± 0.12	1.73 ± 0.51	2.88 ± 0.64	6.94 ± 3.00*

\* =  $p < 0.05$  vs choice latency on correct trials.



Table 4. Erythrocyte AChE activity in 3 mature monkeys treated for 14 days with a 0.01 mg/kg dose of DFP.

Monkey #	Sex	Age (yrs)	Baseline	14 Days	%of Baseline
001	F	12	0.0139	0.0042	30.22
23	M	10	0.0141	0.0027	19.15
215	M	9	0.0119	0.0026	21.85

Activity expressed as  $\mu\text{mol}$  substrate hydrolyzed/min/mg protein in erythrocyte sample.

Figure 8. Effects of DFP administration (0.25 mg/kg/day/14 days) on delayed discrimination accuracy during the Relearning phases of testing. The delayed stimulus discrimination task (DSDT) performance was not altered by DFP exposure. DSDT accuracy during Training (prior to DFP) is presented in the inset.

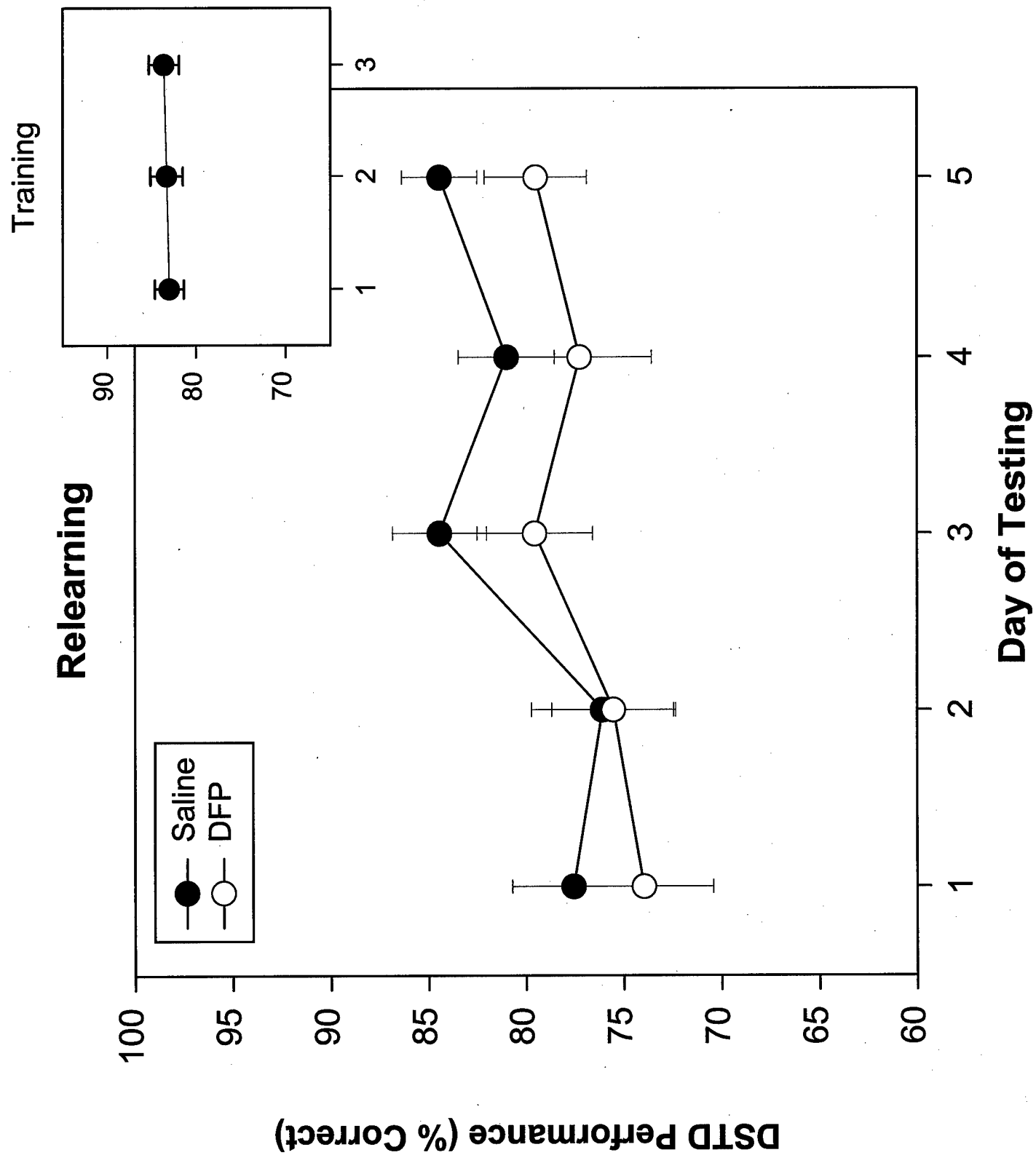


Figure 9. Exposure to DFP (0.25 mg/kg/day/14 days) prior to the onset of acquisition trials produced impairments in ability to locate the hidden platform in the water maze task. The first water maze session began 7 days after completion of the DFP regimen. \* =  $p < 0.05$  vs. saline-controls.

## Aquisition

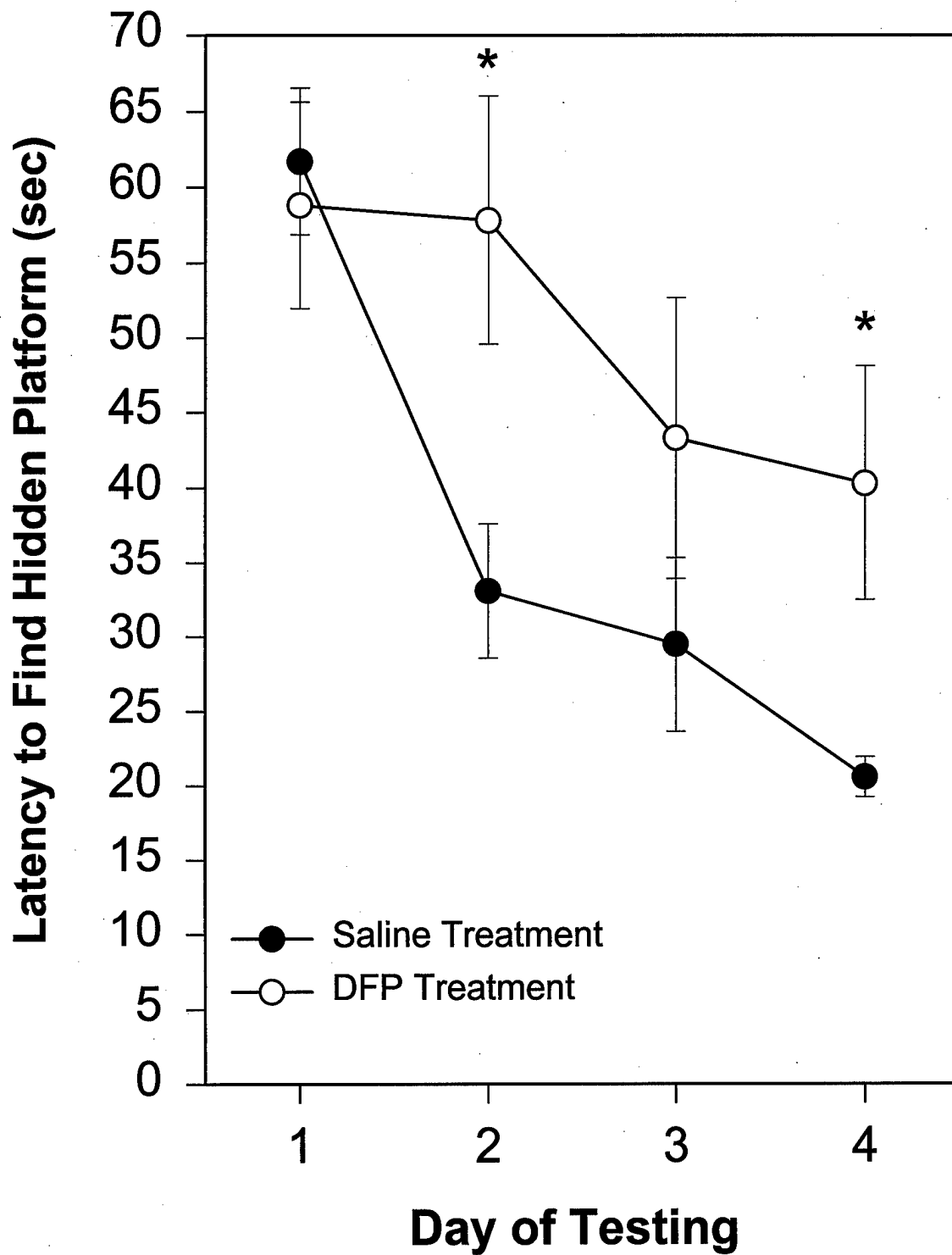


Figure 10. Effects of DFP administration (0.25 mg/kg/day/14 days) on ability to locate a hidden platform in the water maze test during the relearning phase. After different groups of animals completed the initial 4 days of maze training they were administered DFP for 14 consecutive days without maze exposure, allowed to withdraw from DFP exposure for 7 days, and then tested in the water maze for 4 consecutive days in a manner identical to that used during training. Latencies were not altered by exposure to this DFP administration regimen. Latencies to find the platform during initial Training are illustrated in the inset.

## Relearning

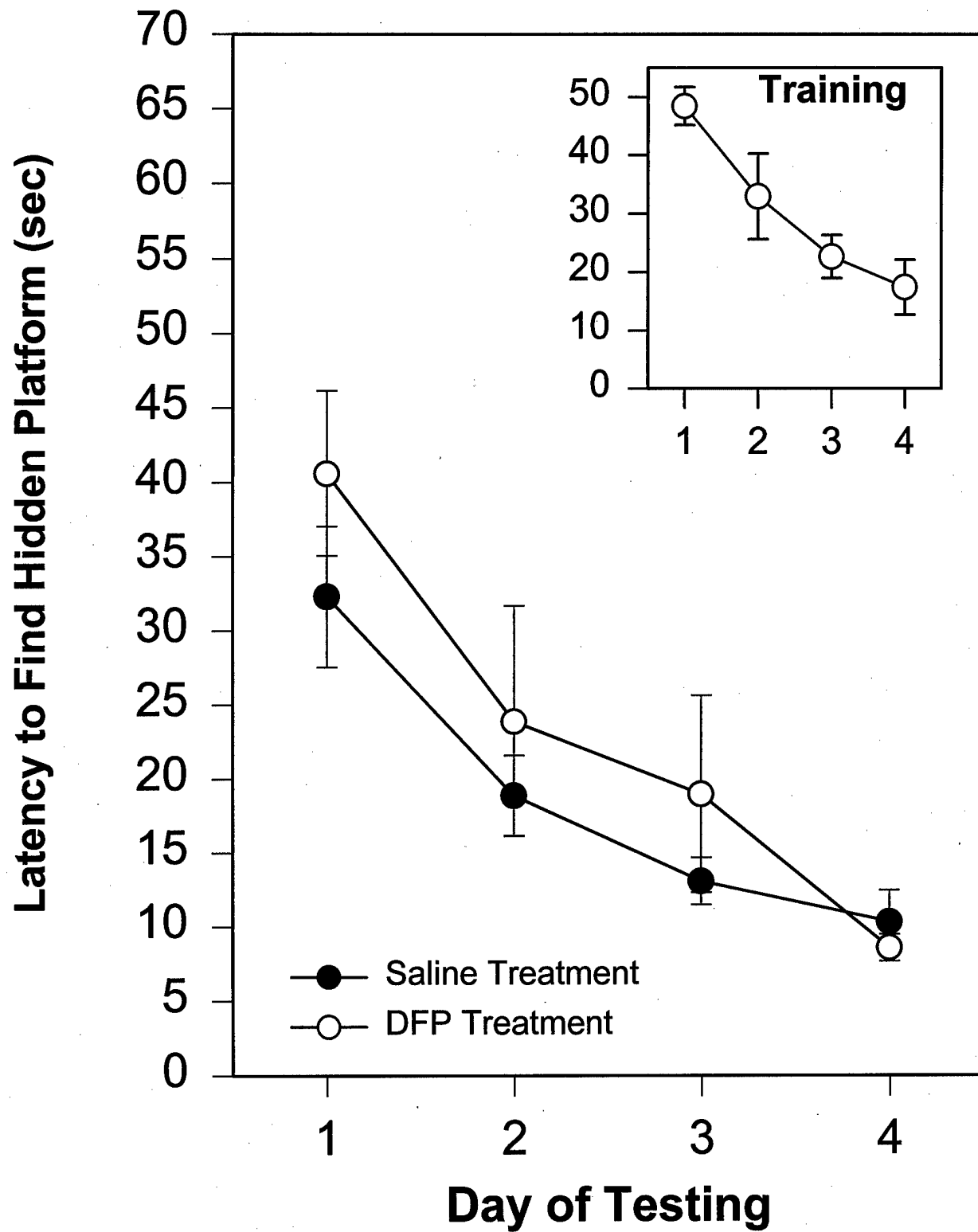


Table 5. [<sup>3</sup>H]QNB Binding to Brain Muscarinic Cholinergic Receptors at Varying Time Intervals After Withdrawal From Chronic Low-Level DFP Treatment

<u>Region</u>	<u>Control</u>	<u>1 day WD</u>	<u>7 days WD</u>	<u>21 days WD</u>
Nucleus Acumbens	100 ± 3.6	91.1 ± 1.6	93.7 ± 7.4	106.6 ± 6.8
Striatum	100 ± 3.9	89.5 ± 1.4	93.6 ± 9.1	103.0 ± 7.8
Parietal Cortex (layers 1-2)	100 ± 3.4	93.5 ± 1.7	82.7 ± 5.7*	90.2 ± 5.7
Parietal Cortex (layers 3-4)	100 ± 4.2	87.5 ± 1.7*	77.5 ± 6.4**	89.9 ± 6.3
Parietal Cortex (layers 5-6)	100 ± 4.7	90.7 ± 1.6	77.9 ± 9.3*	91.8 ± 5.5
Dentate Gyrus	100 ± 2.0	92.7 ± 3.2	90.1 ± 5.0	96.5 ± 2.8
CA1 hippocampus	100 ± 2.5	94.0 ± 3.7	90.4 ± 4.7	97.9 ± 4.7
Superior Colliculus	100 ± 7.3	100.2 ± 4.7	85.0 ± 5.9	110.6 ± 9.1

\* P<0.05; \*\* P<0.01 compared with control means (saline-infused rats); N=6/group.

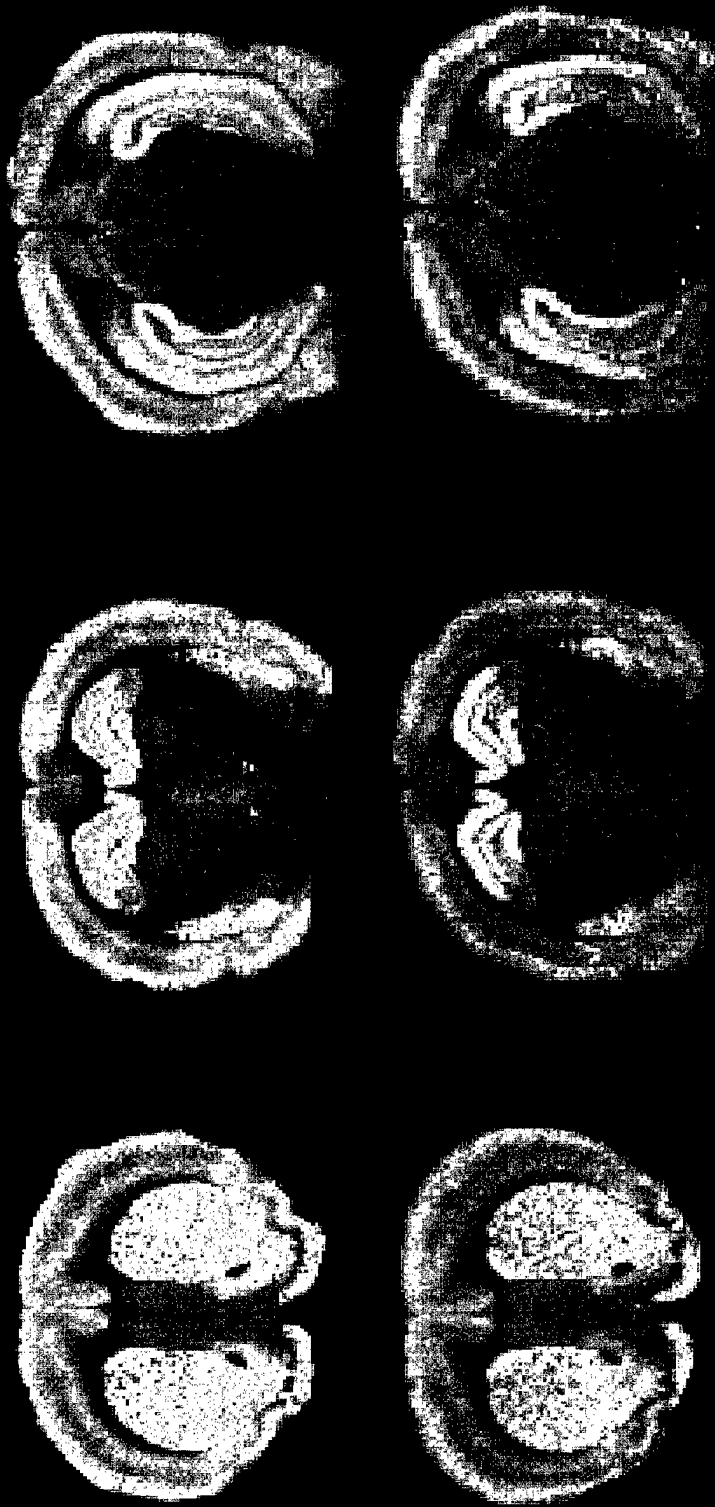


Figure 11. Color enhanced autoradiographs of [ $^3\text{H}$ ]QNB binding to muscarinic cholinergic receptors in different levels of the brain from rostral through caudal (left to right) sections in saline (vehicle) treated rats or in rats that received the chronic DFP regimen. Brains were harvested 1 day after completion of the regimens. Levels of binding are depicted from high through low according to the following color scheme: red>yellow>green>blue>purple. Note the lower level of binding for the parietal cortex for the DFP treated rat.

# $^3\text{H}$ -QNB Binding

Rostral→ Caudal

Saline



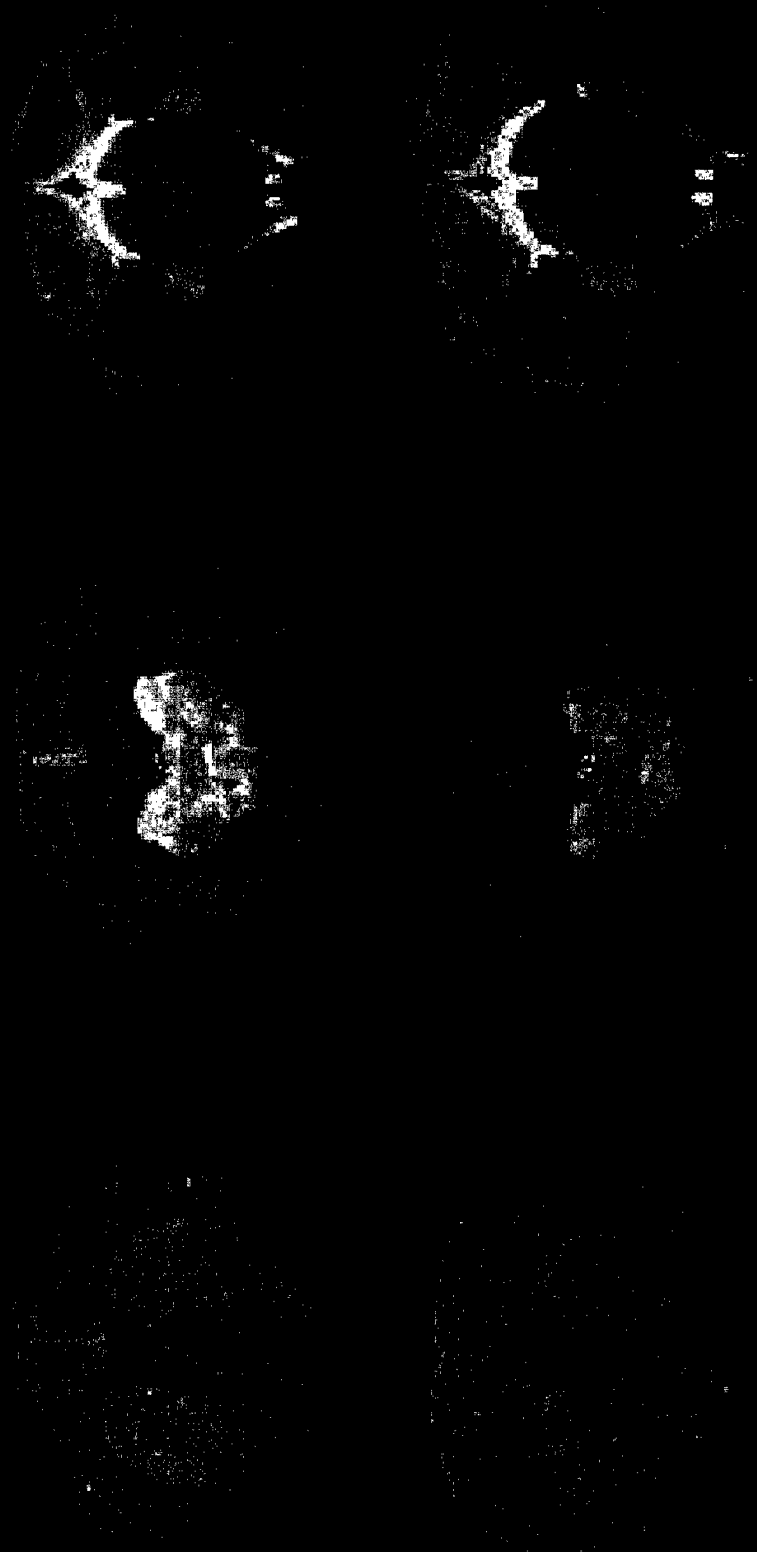
DFP - 1 day  
post-treatment

**Figure 12.** Color enhanced autoradiographs of [ $^3\text{H}$ ]epibatidine binding to high affinity nicotinic cholinergic receptors in different levels of the brain from rostral through caudal (left to right) sections in saline (vehicle) treated rats or in rats that received the chronic DFP regimen. Brains were harvested 1 day after completion of the regimens. Levels of binding are depicted from high through low according to the following scheme: red>yellow>green>blue>purple. Note the low level of binding in the cortical and hippocampal regions (middle sections) for the DFP treated rat.

# $^3\text{H}$ -Epibatidine Binding

Saline

Rostral→ Caudal

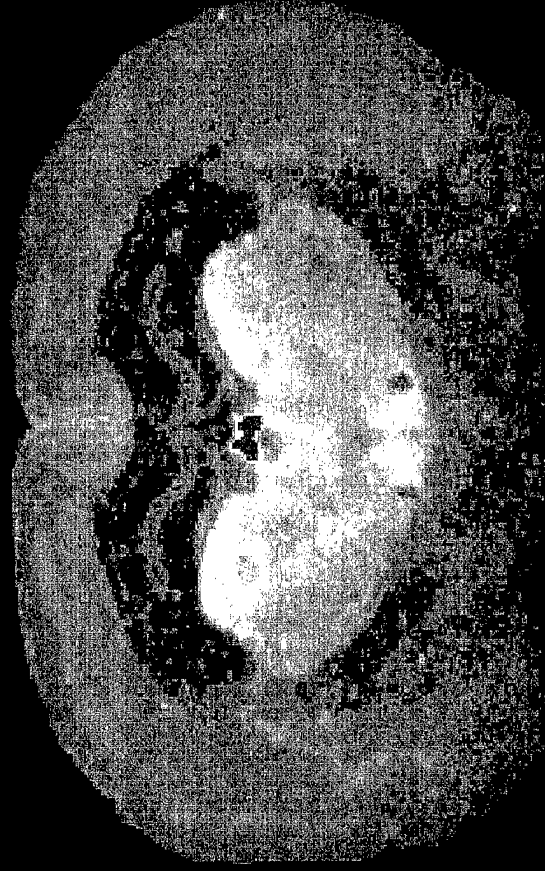
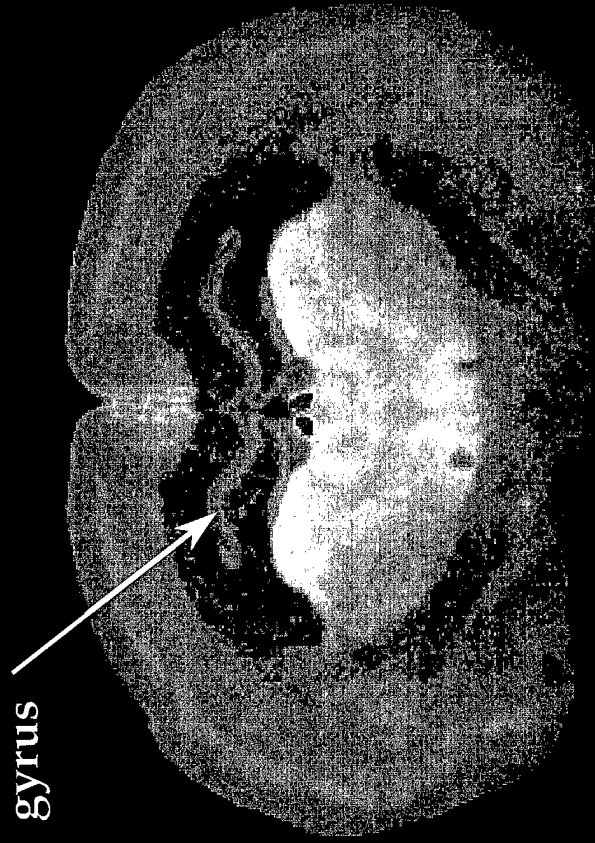


DFP - 1 day  
post-treatment

Figure 13. Color enhanced autoradiographs of [ $^3\text{H}$ ]epibatidine binding to nicotinic cholinergic receptors in the forebrain in saline (vehicle) treated rats, or in rats that received the chronic DFP regimen. Brains were harvested 21 days after completion of the regimens. Levels of binding are depicted from high through low according to the following color scheme: red>yellow>green>blue>purple. Note the lower level of binding in the dentate gyrus of the DFP treated rats when compared with those of the saline treated rats (also see Table 6).

# $^3\text{H}$ -Epibatidine Binding

dentate  
gyrus



Saline

DFP - 21 days  
post-treatment

Table 6. [<sup>3</sup>H]epibatidine Binding to Brain Nicotinic Cholinergic Receptors at Varying Time Intervals After Withdrawal From Chronic Low-level DFP Treatment

<u>Region</u>	<u>Control</u>	<u>1 day WD</u>	<u>7 days WD</u>	<u>21 days WD</u>
Striatum	100 ± 2.9	80.1 ± 8.1	81.7 ± 10.2*	109.1 ± 8.4
Parietal Cortex (layers 4-6)	100 ± 4.4	77.8 ± 14.2	68.9 ± 18.3	105.3 ± 5.4
Parietal Cortex (layer 3)	100 ± 3.2	77.9 ± 10.1*	82.9 ± 10.5	105.2 ± 5.4
Dentate Gyrus	100 ± 9.4	69.2 ± 8.6**	50.9 ± 4.2**	70.3 ± 3.2**
Medial geniculate n.	100 ± 5.2	99.7 ± 10.7	82.4 ± 4.8	94.2 ± 8.7
Superior Colliculus	100 ± 3.9	103.2 ± 9.2	90.8 ± 4.7	97.8 ± 3.9
Subiculum	100 ± 5.0	89.9 ± 7.9	88.2 ± 7.0	90.7 ± 6.5

\* P<0.05; \*\* P<0.01 compared with control means (saline-infused rats); N=6/group.

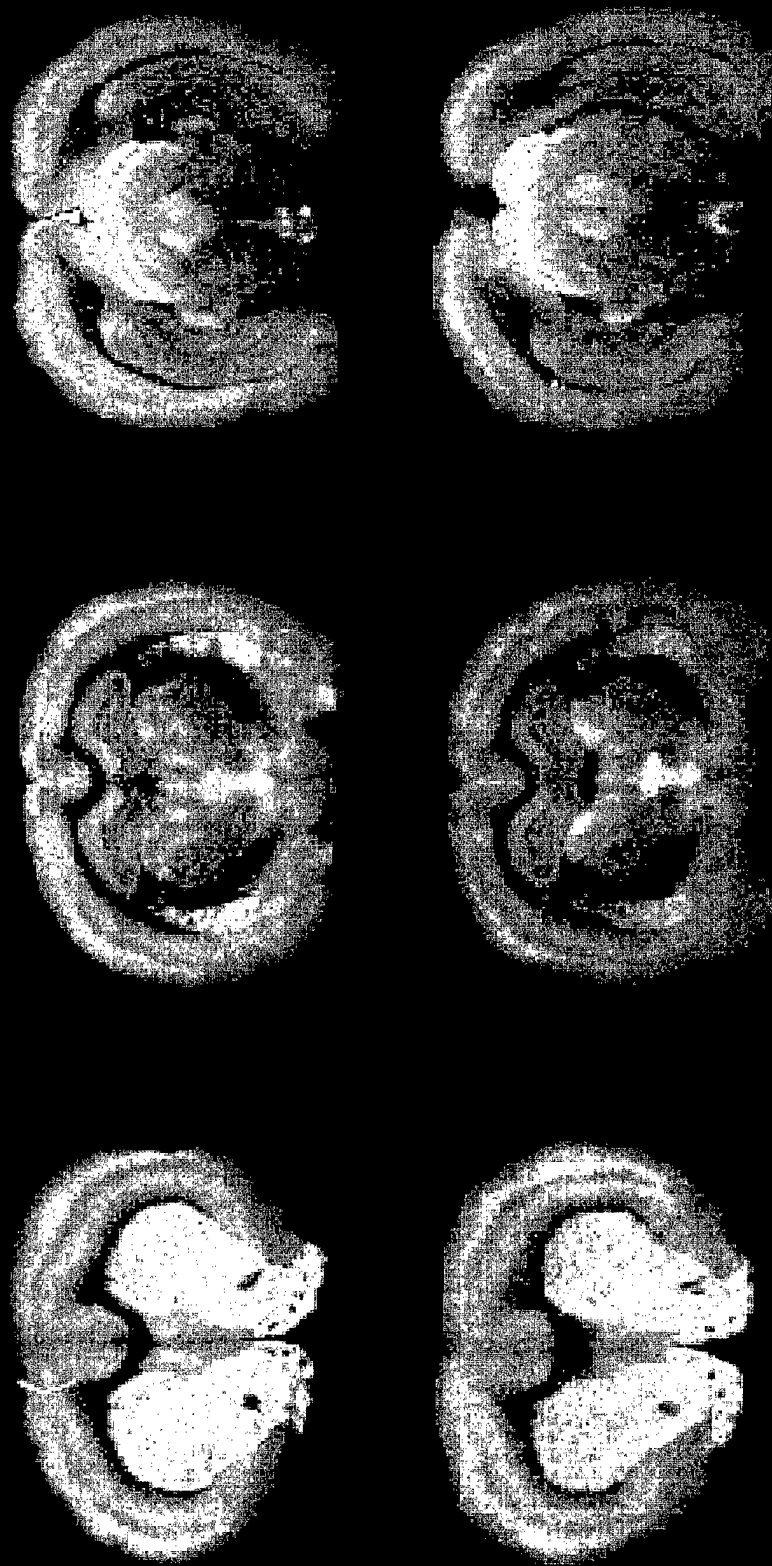
Figure 14. Color enhanced autoradiographs of [ $^3\text{H}$ ]AFDX binding to m2 subtype of muscarinic cholinergic receptors in different levels of the brain from rostral through caudal (left to right) sections in saline (vehicle) treated rats or in rats that received the chronic DFP regimen. Brains were harvested 1 day after completion of the regimens. Levels of binding are depicted from high through low according to the following color scheme: red>yellow>green>blue>purple. Note the lower level of binding in many regions like striatum, hippocampus and parietal cortex of the DFP treated rats when compared with those of the saline treated rats.



# $^3\text{H}$ -AFDX384 Binding

Saline

Rostral→ Caudal



DFP - 1 day  
post-treatment

Table 7. [<sup>3</sup>H]AFDX-384 Binding to Brain Nicotinic Cholinergic Receptors at Varying Time Intervals After Withdrawal From Chronic Low-level DFP Treatment

<u>Region</u>	<u>Control</u>	<u>1 day WD</u>	<u>7 days WD</u>	<u>21 days WD</u>
Striatum	100 ± 10.1	56.8 ± 7.7**	61.1 ± 11.0*	77.1 ± 11.3*
Nucleus Accumbans	100 ± 7.9	62.0 ± 9.5**	63.9 ± 9.7*	105.0 ± 12.4
Dentate Gyrus	100 ± 10.6	69.9 ± 10.1*	87.9 ± 12.9	69.2 ± 10.6
CA1 hippocampus	100 ± 7.2	57.0 ± 8.3**	74.3 ± 13.5	94.6 ± 10.8
Parietal Cortex (layers 1-2)	100 ± 9.3	83.1 ± 7.9	75.3 ± 10.1	90.1 ± 5.4
Parietal Cortex (layers 3-4)	100 ± 12.4	73.0 ± 9.9*	71.0 ± 13.1	85.5 ± 5.8
Parietal Cortex (layers 5-6)	100 ± 9.8	76.0 ± 8.9*	69.9 ± 10.7	82.7 ± 12.0
Superior Colliculus	100 ± 4.1	92.5 ± 8.2	96.1 ± 4.3	92.0 ± 6.4
Subiculum	100 ± 14.6	49.6 ± 14.6**	36.0 ± 7.37**	42.3 ± 2.8**

\* P<0.05; \*\* P<0.01 compared with control means (saline-infused rats); N=6/group.

Figure 15. Time course for the changes in brain cerebral cortical or hippocampal levels of acetylcholinesterase (AChE) activity, neuronal nicotinic cholinergic ( $[^3\text{H}]$ epibatidine) receptors, and muscarinic cholinergic ( $[^3\text{H}]$ QNB and  $[^3\text{H}]$ AFDX) receptors after completion of a chronic low-level regimen of DFP (0.25 mg/kg/day/14 days). Note that there was a significant delay in the recovery of hippocampal AChE levels relative to cortical levels. Also, nicotinic receptor expression in the hippocampus was significantly reduced for up to 3 weeks after DFP exposure. Changes in muscarinic receptors as measured by  $[^3\text{H}]$ QNB binding were of smaller magnitude than those for nicotinic receptors, and there was less of a regional difference between the time courses. However,  $[^3\text{H}]$ AFDX binding specific for M2 receptor revealed a significant reduction in this subtype up to 3 weeks after DFP exposure similar to that of the epibatidine binding.

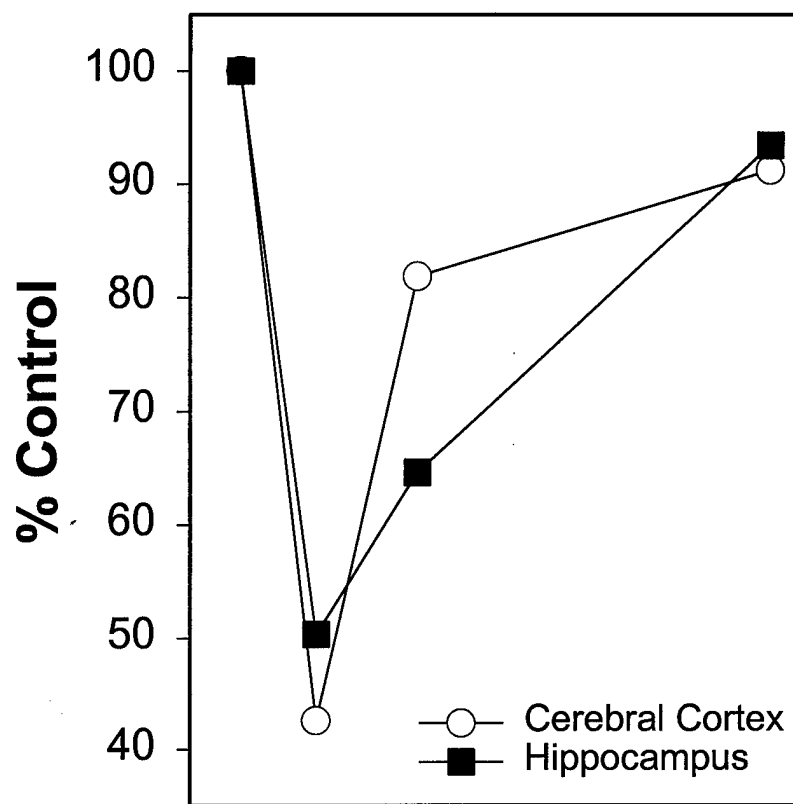
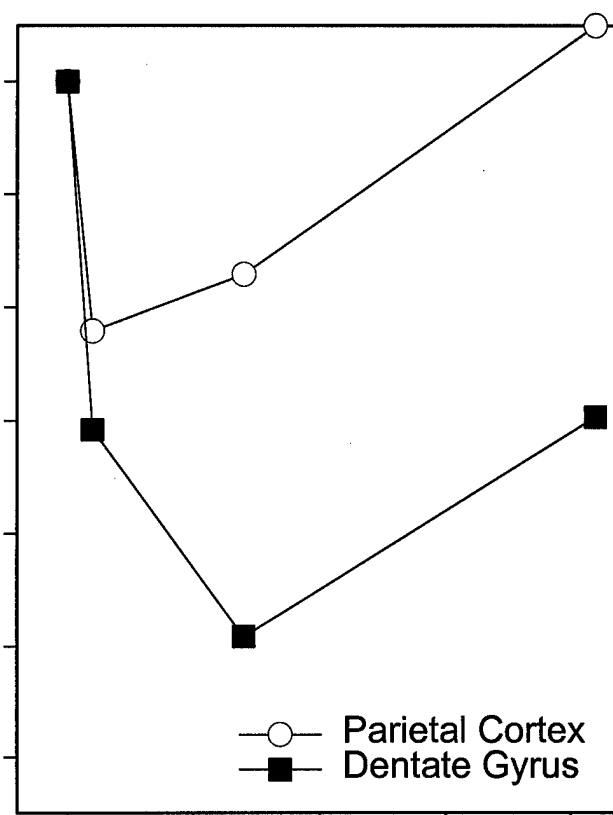
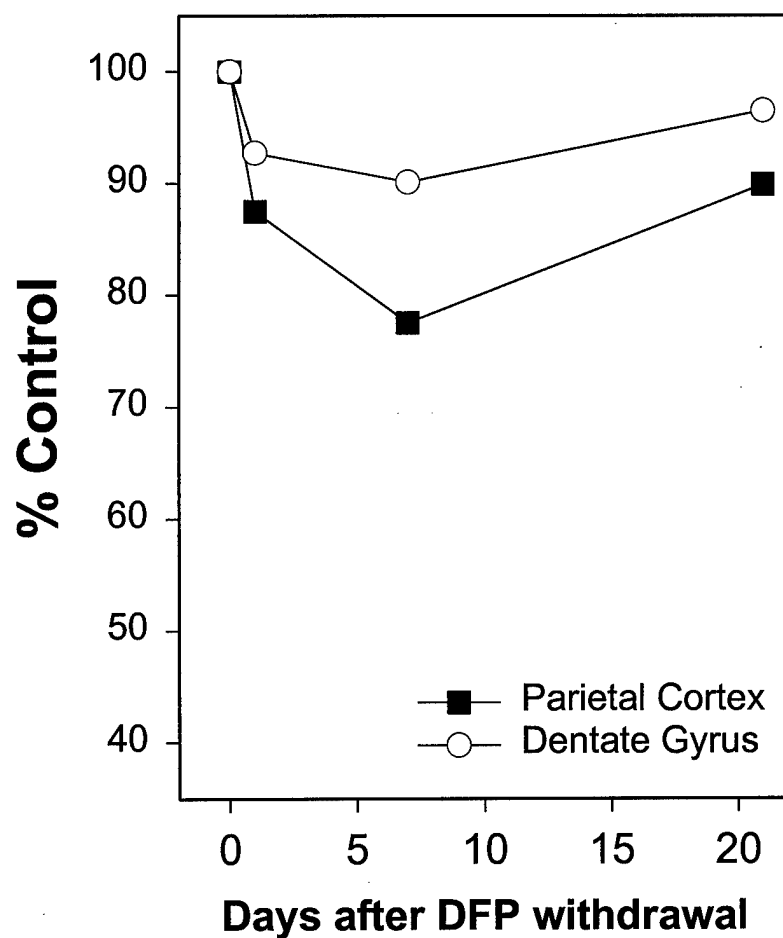
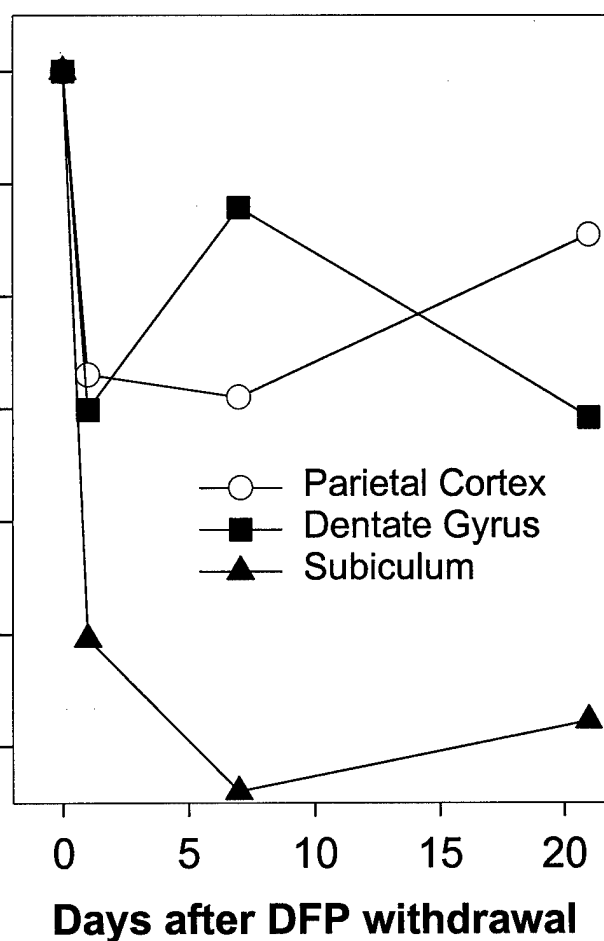
**ACHE Activity****[<sup>3</sup>H]Epibatidine Binding****[<sup>3</sup>H]QNB Binding****[<sup>3</sup>H]AFDX 384 Binding**

Figure 16. Effect of pre-session injection of nicotine (Nic, 0.5 or 1.0 mg/kg, s.c.) on water maze performance (as measured by swim latency) of rats previously treated with either a chronic low-level regimen of DFP (DFP/Sal; 0.25 mg/kg/day/14 days) or control saline (Sal/Sal) regimen. Water maze testing was initiated 2 weeks after completion of the DFP or saline regimen. Both doses of nicotine significantly reversed the DFP-induced impairment in task acquisition as measured by swimming latencies (time required to locate the hidden platform). There was a significant main "drug" effect by ANOVA,  $F(3,227)=4.25$ ,  $P=0.006$ . The DFP/SAL group was significantly different from the other 3 groups;  $P<0.05$  (Student-Newman-Keuls multiple comparison test).

# Water Maze Performance

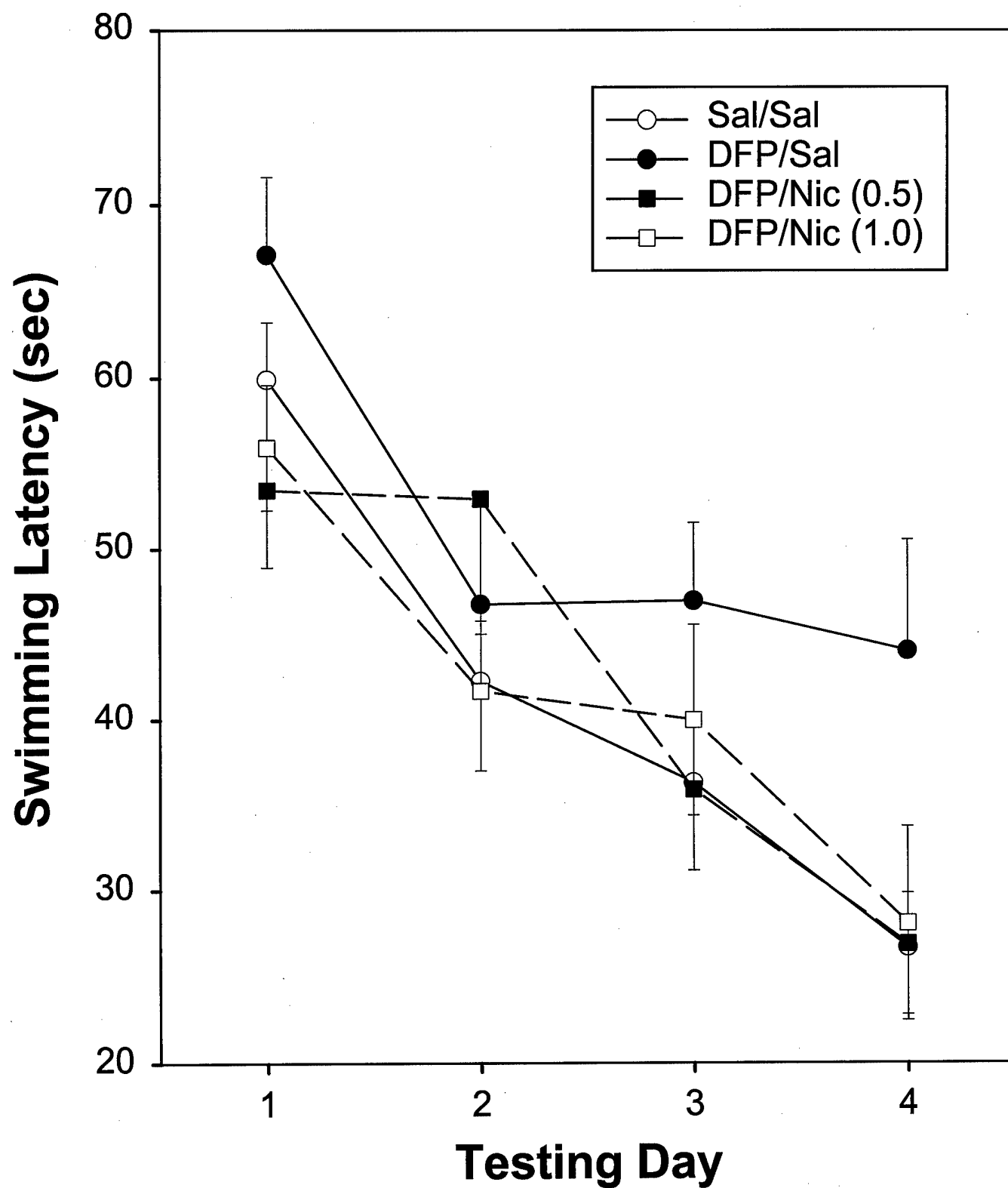


Figure 17. Effect of pre-session injection of nicotine (Nic, 0.5 or 1.0 mg/kg, s.c.) on water maze performance (as measured by swim distance) of rats previously treated with either a chronic low-level regimen of DFP (0.25 mg/kg/day/14 days) or saline regimen. Water maze testing was initiated 2 weeks after completion of the DFP or saline regimen. Both doses of nicotine significantly reversed the DFP-induced impairment in task acquisition as measured by swimming distance (distance traveled to locate the hidden platform). There was a significant main “drug” effect by ANOVA,  $F(3,227)=4.25$ ,  $P<0.001$ . The DFP/SAL group was significantly different from the other 3 groups;  $P<0.05$  (Student-Newman-Keuls multiple comparison test).

# Water Maze Performance

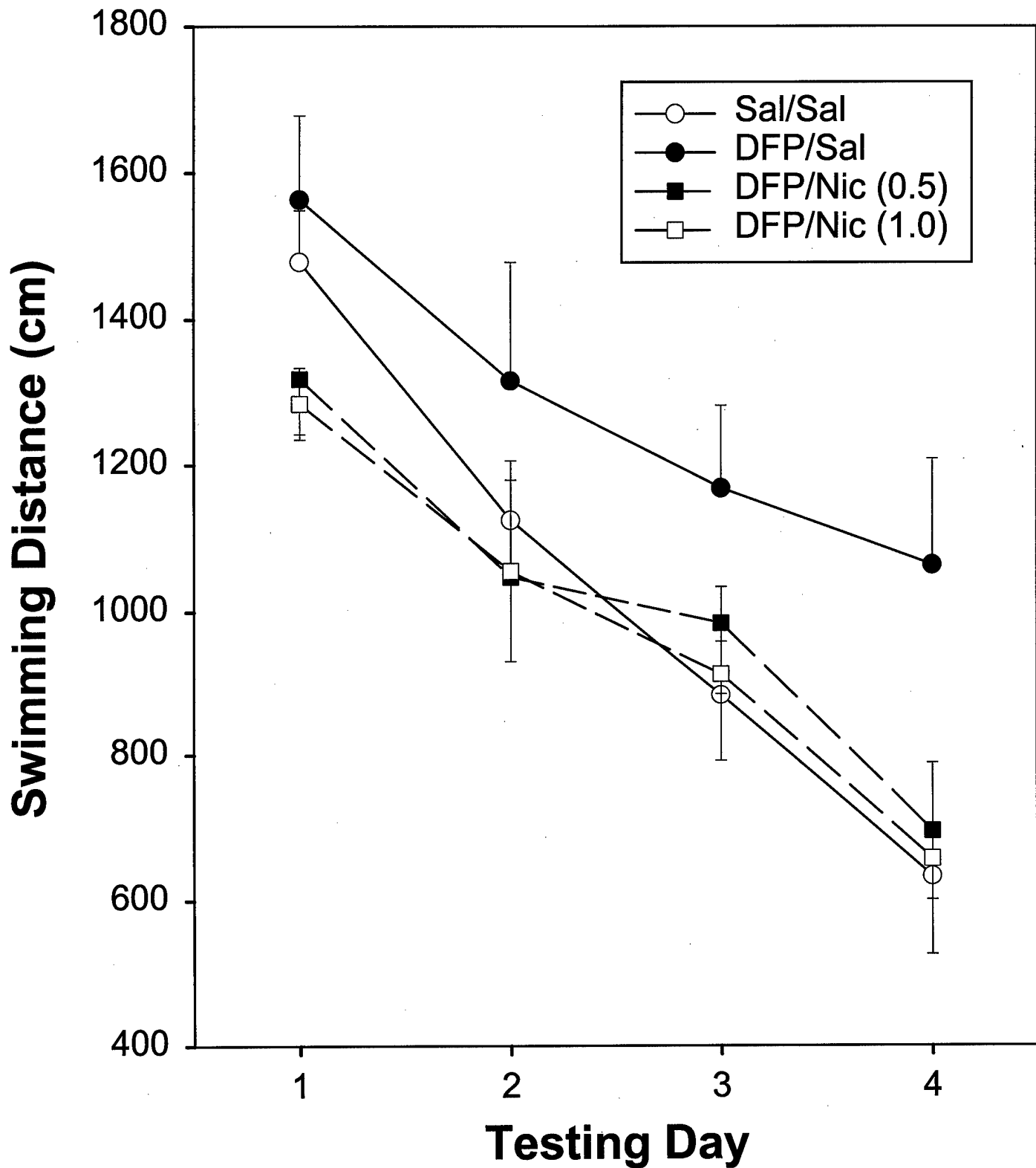
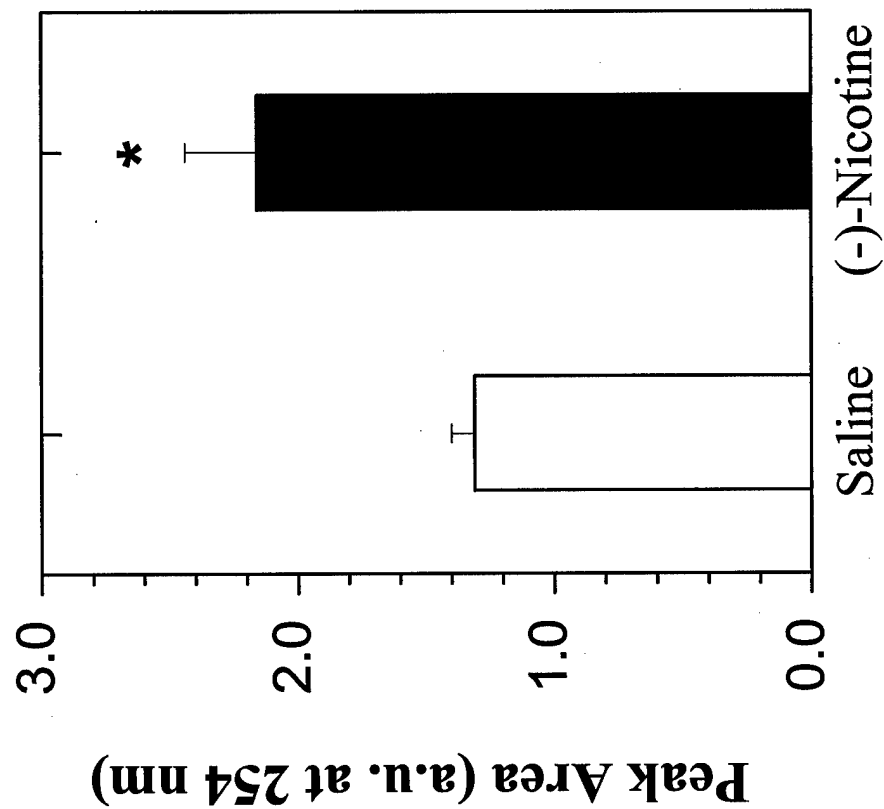




Figure 18. Detection by ion-exchange chromatography of vesicular acetylcholine transporter (VACht) cDNA abundance in the hippocampus and cerebral cortex following (-)-nicotine administration. Endogenous mRNA was amplified using RT-PCR. \* =  $P < 0.05$  vs saline-treated animals.

## VACht cDNA

### Cerebral Cortex



### Hippocampus

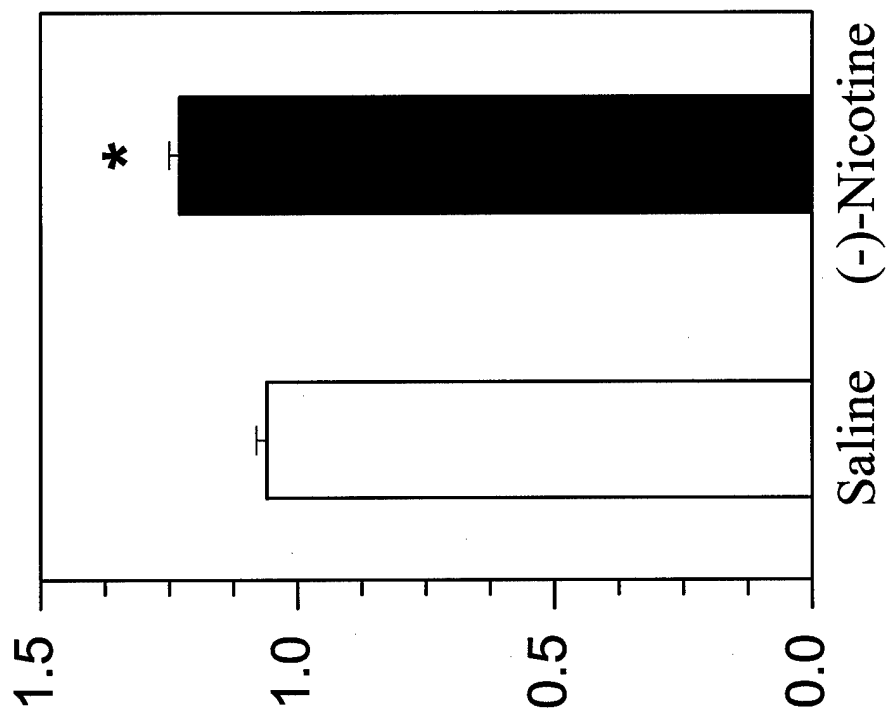


Figure 19. The effect of daily oral administration of 200 mg/kg of CP for 14 days prior to, and during 6 days of water maze testing on the scopolamine-induced (0.5 mg/kg, i.p.) performance deficits in rats. A. Mean swimming latencies. B. Mean swimming distance. Each value represents the mean  $\pm$  s.e.m. of 6 rats. (■) - Chronic treatment: 14 days of CP vehicle (peanut oil); Pre-test treatment: scopolamine alone. (▼) - Chronic treatment: CP; Pre-test treatment: scopolamine and CP; (●) - Chronic treatment: vehicle; Pre-test treatment: saline, i.p. (▲) - Chronic treatment: CP; Pre-test treatment: CP. Each pre-test drug regimen was administered 20 min before maze testing. The scopolamine alone group was significantly different from each of the other groups ( $p < 0.05$ ).

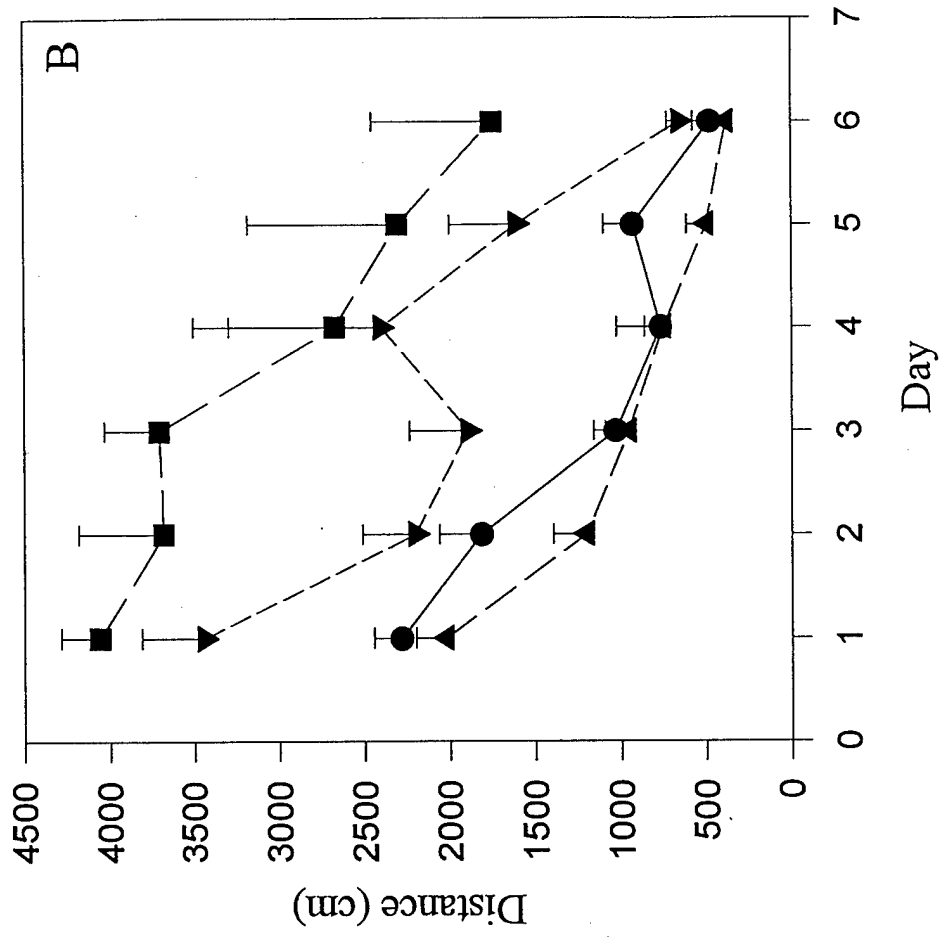
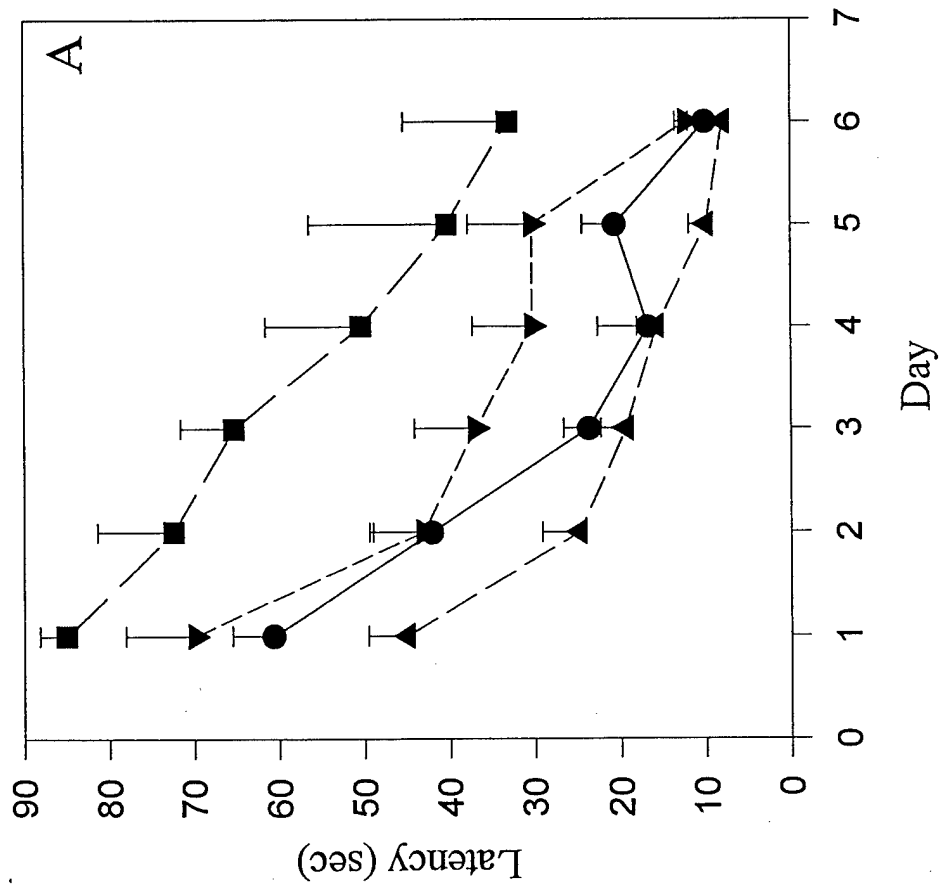


Table 8. Effect of CP on scopolamine treated and untreated rats in open-field locomotor activity performance.

a). Chronic study

Activity	Con	Sco	CP	CP+S
Horizontal activity	9187 ± 994	14830 ± 1456*	12152 ± 907	13735 ± 1390*
Movement time (sec)	515 ± 57	795 ± 81*	623 ± 45	712 ± 72
Number of stereotypy	220 ± 15	266 ± 19*	272 ± 7*	275 ± 9*
Vertical Activity	2647 ± 261	3873 ± 359	3273 ± 284	3607 ± 350

\* Significantly different than control (C) rats ( $p < 0.05$ ).

Values are mean ± s.e.m. of 6 rats.

b). Acute study

Activity	Con	Sco	CP	CP+S
Horizontal activity	11343 ± 1005	22284 ± 2607*	10254 ± 629	24288 ± 2598*
Movement time (sec)	886 ± 86	1429 ± 70*	811 ± 49	1473 ± 43*
Number of stereotypy	220 ± 13	308 ± 9*	181 ± 9	299 ± 10*
Vertical Activity	2243 ± 298	2268 ± 3651	2319 ± 453	3198 ± 606

\* Significantly different than control (Con) and CP treated rats ( $p < 0.05$ ).

No significant differences are present between either control and CP group or scopolamine (Sco) and CP + Scopolamine treated groups.

Values are mean ± s.e.m. of 6 rats. Figure 20. The effect of daily oral administration of CP for 14 days prior to, and during 6 days of water maze testing on the scopolamine-induced (0.5 mg/kg, i.p.) performance deficits in rats. Only days 2 and 3 of maze testing are presented. Each value represents the mean ± s.e.m of 6-12 rats. **(C)** - Chronic treatment: CP vehicle (peanut oil); Pre-test treatment: saline, i.p. **(S)** - Chronic treatment: 14 days of CP vehicle (peanut oil); Pre-test treatment: scopolamine alone. **(50)** - Chronic treatment: 50 mg/kg CP; Pre-test treatment: scopolamine and 50 mg/kg of CP. **(200)** - Chronic treatment: 200 mg/kg CP; Pre-test treatment: scopolamine and 200 mg/kg CP. **(400)** - Chronic treatment: 400 mg/kg CP; Pre-test treatment: scopolamine and 400 mg/kg CP.. \*The scopolamine alone group was significantly different from each of the other groups ( $p < 0.05$ ).

**Figure 20.** The effect of daily oral administration of CP for 14 days prior to, and during 6 days of water maze testing on the scopolamine-induced (0.5 mg/kg, i.p.) performance deficits in rats. Only days 2 and 3 of maze testing are presented. Each value represents the mean  $\pm$  s.e.m of 6-12 rats. **(C)** - Chronic treatment: CP vehicle (peanut oil); Pre-test treatment: saline, i.p. **(S)** - Chronic treatment: 14 days of CP vehicle (peanut oil); Pre-test treatment: scopolamine alone. **(50)** - Chronic treatment: 50 mg/kg CP; Pre-test treatment: scopolamine and 50 mg/kg of CP. **(200)** - Chronic treatment: 200 mg/kg CP; Pre-test treatment: scopolamine and 200 mg/kg CP. **(400)** - Chronic treatment: 400 mg/kg CP; Pre-test treatment: scopolamine and 400 mg/kg CP.. \*The scopolamine alone group was significantly different from each of the other groups ( $p < 0.05$ ).

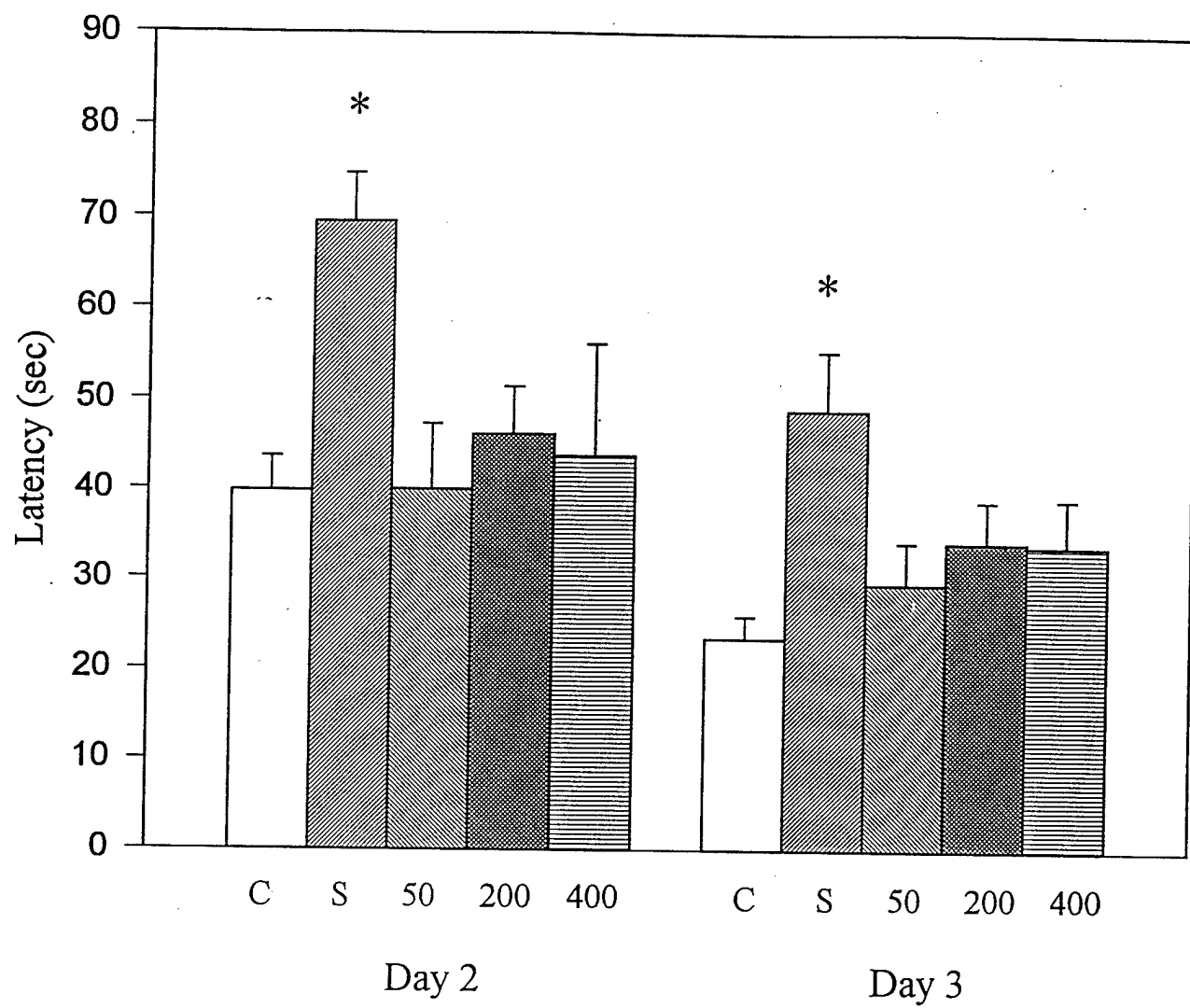


Figure 21. The effect acute administration of 200 mg/kg of CP prior to, and during 6 days of water maze testing on the scopolamine-induced (0.5 mg/kg, i.p.) performance deficits in rats. A. Mean swimming latencies, B. Mean swimming distance. Each value represents the mean  $\pm$  s.e.m. of 6 rats. (■) - scopolamine alone. (▼) - scopolamine and CP; (●) - saline, i.p. (▲) - CP alone. Each pre-test drug regimen was administered 20 min before maze testing. The scopolamine alone and the scopolamine + CP groups were significantly different from the saline and CP alone groups ( $p < 0.05$ ).



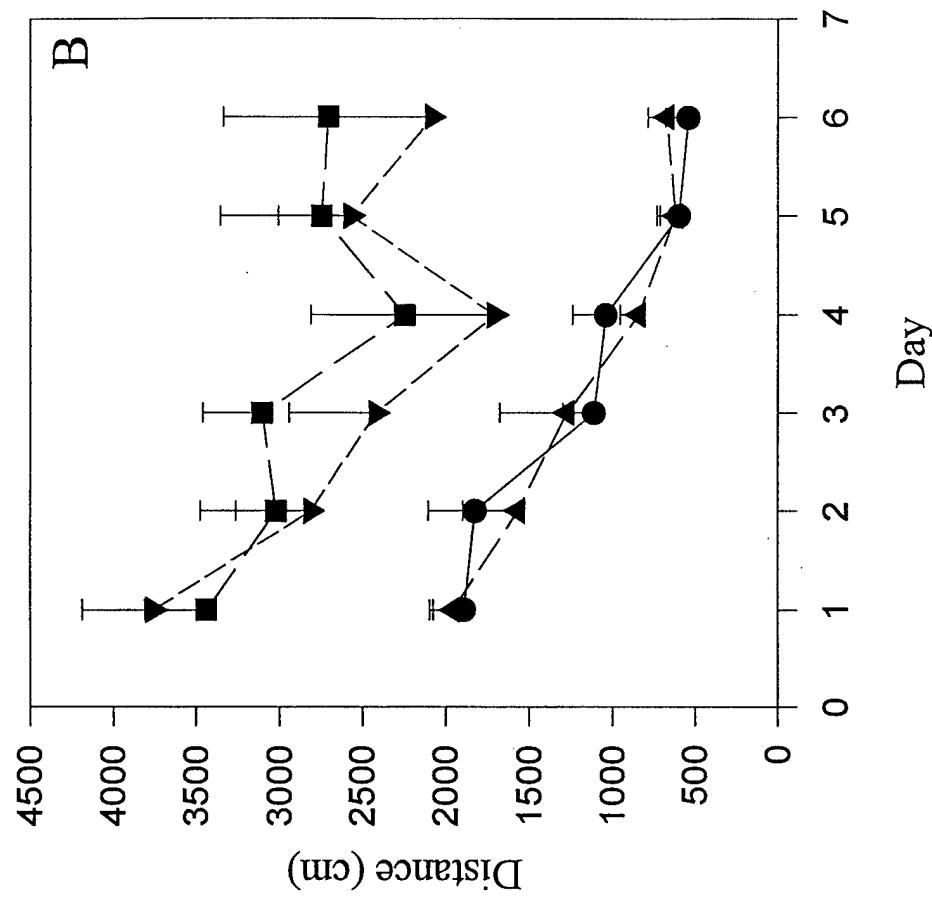
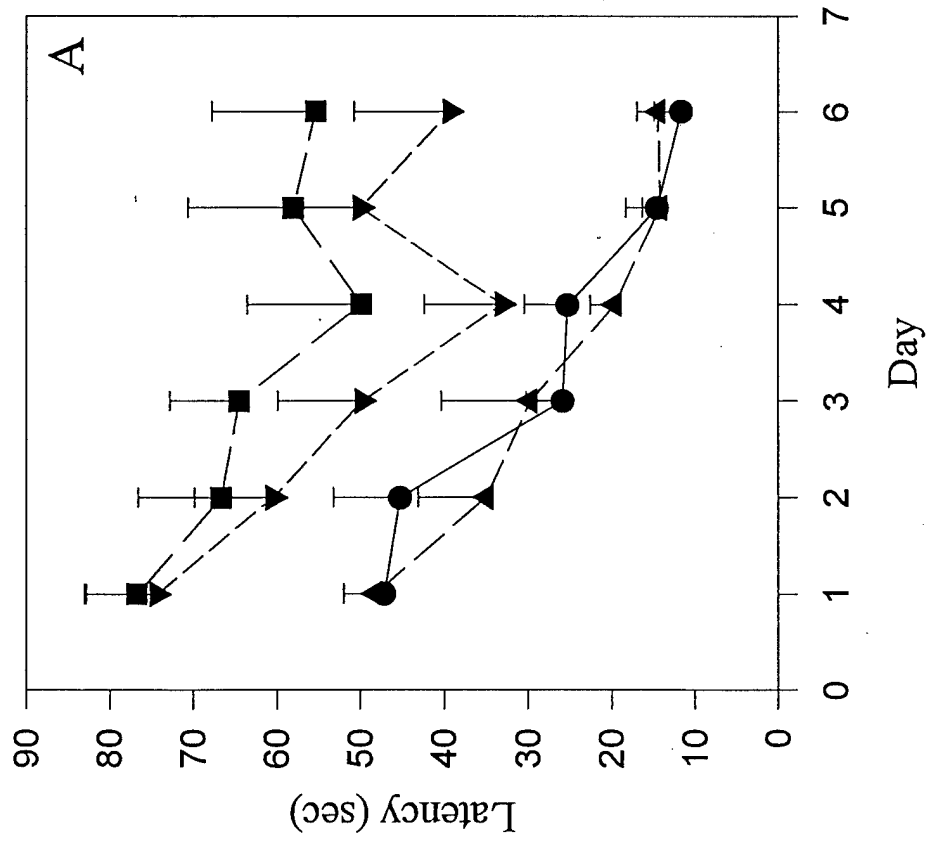


Figure 22. The effect of chronic administration of DFP alone or in combination with pyridostigmine bromide (PB) on olfactory reactivity. In four experimental groups rats received either saline vehicle (SAL), PB (0.40 mg/kg, p.o., t.i.d.), DFP (250 µg/kg, s.c., once daily), or a combination of both PB and DFP (PB/DFP) for 7 days. DFP-containing regimens, and in particular the combined PB/DFP regimen reduced rearing behavior after 1-3 days of exposure. Rearing in animals that received PB or DFP alone began to recover to near-control levels after 5-6 days of exposure. \* =  $P < 0.05$  compared with SAL group.

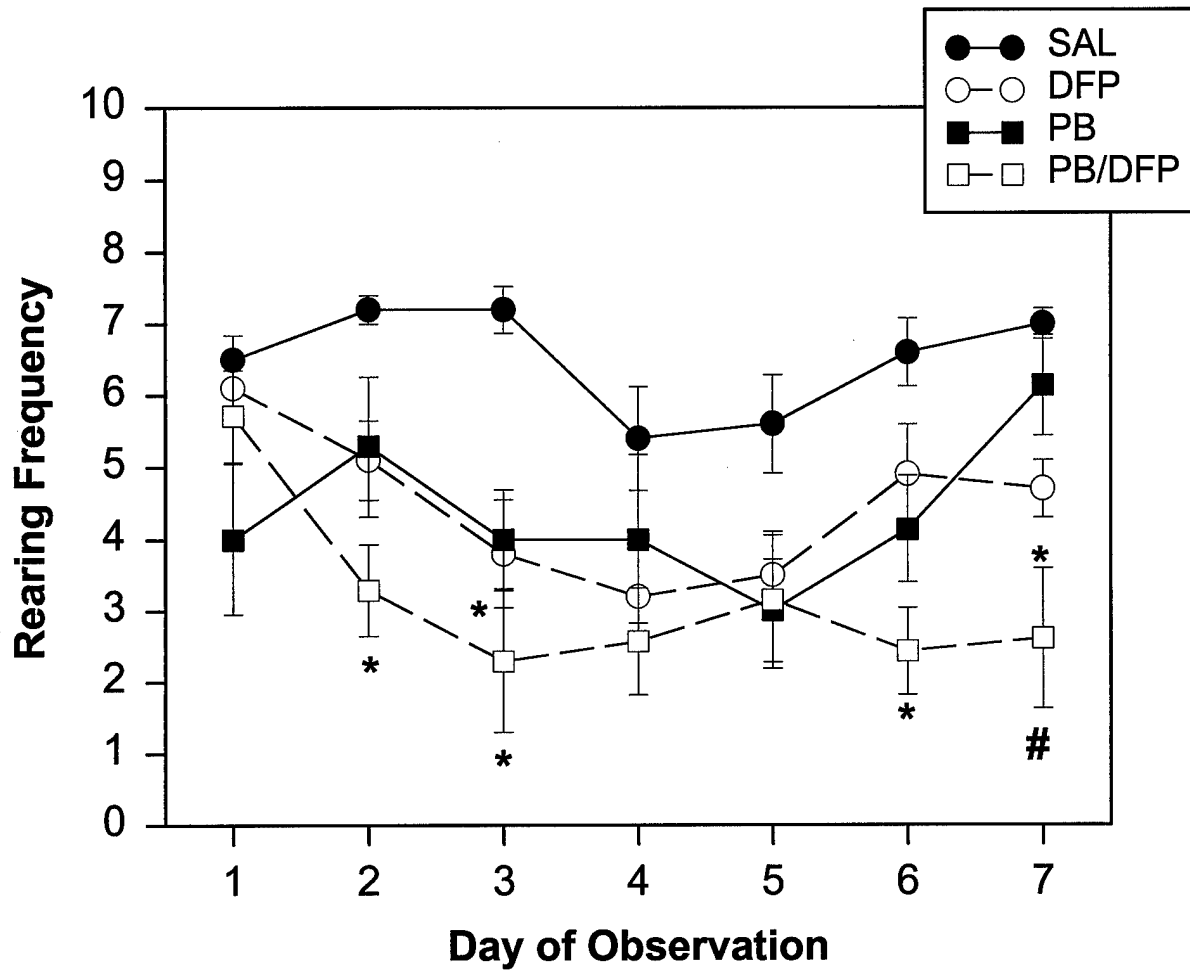
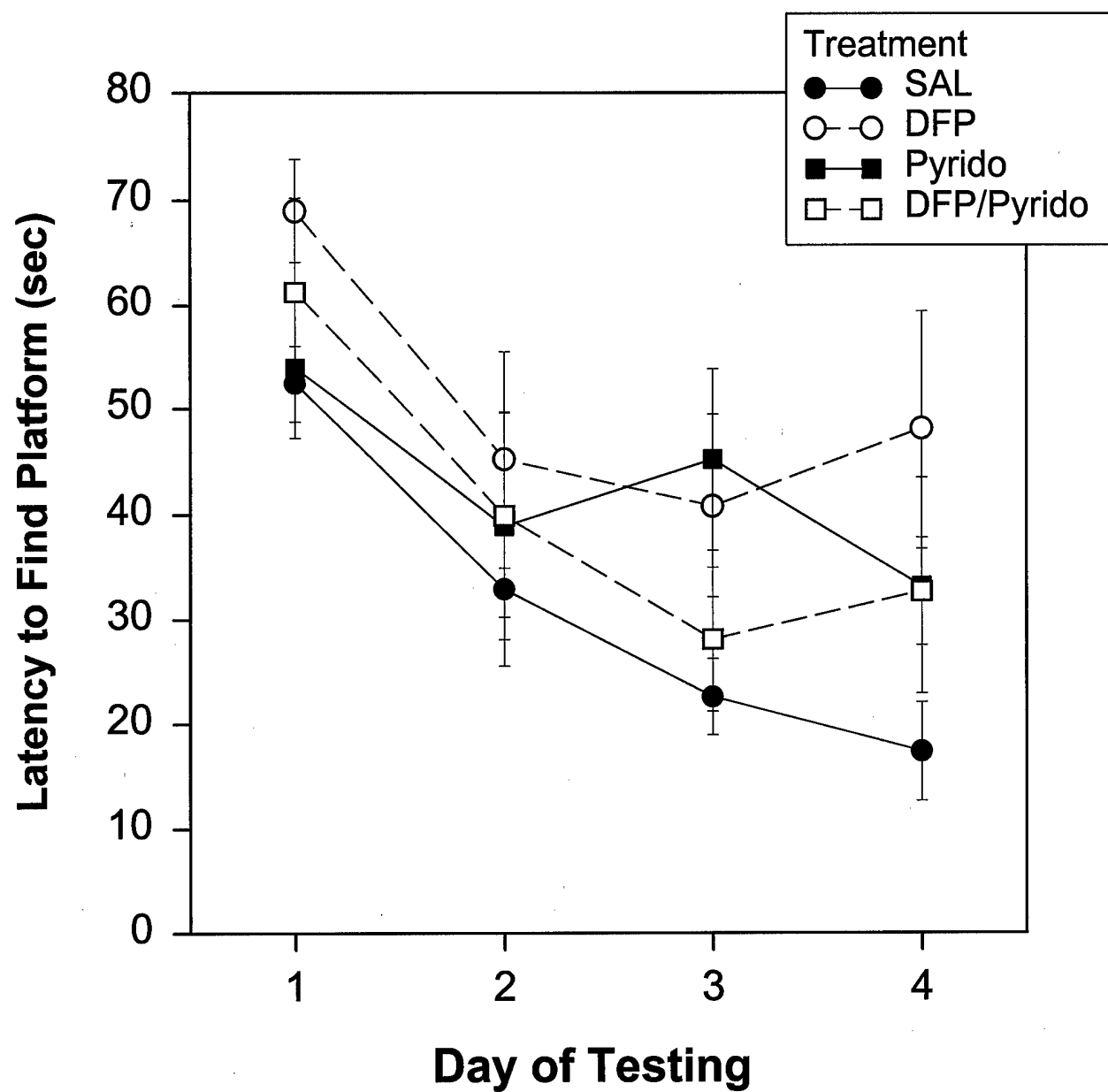


Figure 23. The effect of chronic administration of DFP alone or in combination with pyridostigmine bromide (PB) on water maze testing. In four experimental groups rats received either saline vehicle (SAL), PB (0.40 mg/kg, p.o., t.i.d.), DFP (0.25 mg/kg, s.c., once daily), or a combination of both PB and DFP (PB/DFP) for 7 days. Testing was initiated 1 week after exposure. Only the DFP group was significantly different from the SAL group,  $P < 0.05$ .



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## **Scientific News Article**

"The Big Easy Serves Up a Feast To Visiting Neuroscientists", *Science*, 278: 1404, 1997.

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